Long View for STIC Online Catalog

Your Search: **Title Browse = Pathology**
Displaying Record: **1 of 1**

Title	Pathology
Dates of Pub	v. 29, no. 1 (1997)-v. 34, no. 6 (2002)
Notes	"Available on ADONIS, v. 29, no. 1 (1997)-v. 34, no. 6 (2002)

Biotechnology and Chemical Library	ADONIS	Available

Email katherine.arendt@uspto.gov with any questions or comments about the STIC Online Catalog.

Lippincott Williams & Wilkins

Current Opinion in Neurology

Volume 16(3) June 2003 pp 289-297

Management of multiple sclerosis: current trials and future options

[Demyelinating diseases]

Noseworthy, John H.

Department of Neurology, Mayo Medical School, Clinic and Foundation, Rochester, Minnesota, USA
Correspondence to J.H. Noseworthy, MD, Department of Neurology, Mayo Clinic Building, W8A, 200 First St, SW, Rochester, MN 55905, USA Tel: +1 507 284 2675; fax: +1 507 266 0178; e-mail: Noseworthy.john@mayo.edu

AbbreviationsEDSS: Expanded Disability Status ScaleGA: glatiramer acetateMRI: magnetic resonance imagingMS: multiple sclerosisMSFC: Multiple Sclerosis Functional CompositeNAB: neutralizing antibodyRCT: randomized controlled trialRRMS: relapsing/remitting multiple sclerosisSPMS: secondary progressive multiple sclerosis

Outline

Graphics

Abstract

Purpose of the review: The present review of multiple sclerosis (MS) therapeutic trials published in 2002 is intended to assist the reader in understanding the most current advances in the care of their patients.

Recent findings: A substantial number of pivotal and preliminary reports continue to demonstrate encouraging new evidence that advances are being made in the care of patients with MS. Several short-term studies in relapsing/remitting MS have demonstrated that it is possible to complete head-to-head comparison trials of active agents in MS (e.g. without a placebo control group). The findings of these trials remain open to interpretation and have generated considerable controversy, as expected. A phase 3 trial [the International MS Secondary Progressive Avonex Controlled Trial (IMPACT)] became the fourth study of the [beta] interferons (interferon-[beta]-1a, in this case) to demonstrate a partial effect on disease activity in secondary progressive MS. Two trials demonstrated apparent partial efficacy for the anthrecenedione mitoxantrone in active and progressive MS. Disappointing results were announced for a number of large pivotal trials, although those results have not yet been published (e.g. oral glatiramer acetate in relapsing/remitting MS, glatiramer acetate in primary progressive MS, and intravenous immunoglobulin in secondary progressive MS).

Summary: The MS research community needs to determine how best to address two key unanswered questions. Is late clinical deterioration often or invariably tied to the initial inflammatory/demyelinating phase of the disease? What is the optimal research design to address whether current and future experimental strategies affect the later phases of MS (e.g. does early treatment delay or prevent clinical disability)?

Introduction

In recent years, since the pivotal trials of the [beta] interferons [1,2] and glatiramer acetate (GA) [3], with a few exceptions the performance of candidate multiple sclerosis (MS) treatments in randomized controlled trials (RCTs) has been disappointing [4•]. Nonetheless, there is an astonishing array of therapeutic strategies being evaluated, and the opportunities for future advances seem promising [5]. In this 'ABC era', physicians and patients face a choice between one of several approved [beta] interferons (i.e. interferon-[beta]-1b and interferon-[beta]-1a) or GA, with sparse controlled, comparative evidence to guide such decisions. Each of these agents reduces clinical and magnetic resonance imaging (MRI) indicators of inflammatory disease activity (clinical relapses, new and active MRI lesions), although for most of the published studies the period of follow up is short (<3 years [6]). There are immunologic data to support the opinion that the course of disease may be altered by treatment, and there is lively debate regarding which agent is most effective. Relatively small differences in apparent effects on clinical behavior, imaging, and immunologic markers sway opinion. Additionally, the adverse event profiles, the availability of multiple dosing options, the relative convenience of dosing routes and schedules, and the propensity to induce neutralizing antibodies (NABs) are all cited in discussions about treatment preferences. It appears that some MS specialists have a strong preference for one agent whereas others have relative equipoise. Either way, one hopes that treating physicians involve their patients in treatment discussions, carefully reviewing what is known about safety and efficacy.

This year a number of trials directly compared the clinical and MRI outcomes between various interferons. Each was designed to reach a timely decision about a short-term benefit. As outlined below, these studies demonstrating short-term treatment advantages of one agent or one dosing strategy have generated considerable controversy. None of the studies was designed to answer unequivocally whether there is an important and sustained treatment advantage that will be durable (e.g. that will persist throughout the prolonged course of this chronic illness). Regrettably, none of the studies was fully blinded, presumably because those conducting the studies judged that this design element was either not necessary or not feasible (e.g. because of common, recognizable adverse effects or dosing schedules), depending on the individual trial. Essentially, the studies could only hope to determine whether one agent (or one dose) is preferable to another for the short period of observed follow up. It is currently unclear whether the outcomes of those brief trials will influence prescribing patterns or whether most physicians will continue to view the available, 'partially effective' drugs as being approximately comparable and will await agents of greater therapeutic benefit or evidence of prolonged benefit.

Completed randomized clinical trials and preliminary new data

In the last year, several phase 3 trials and a larger number of preliminary trials have been reported. The trials reported to this point have not been designed to be of sufficient duration, however, to demonstrate unequivocal evidence that the long-term outcome of this chronic illness has been favorably altered although short-term evidence provides considerable reason for guarded optimism.

Relapsing/remitting multiple sclerosis

Two striking examples of recently completed comparison trials include the INCOMIN (INdependent COMparison of INterferon) and EVIDENCE (EVidence of Interferon Dose-response: European North American Comparative Efficacy; Table 1). In the INCOMIN trial [7••], 188 relapsing/remitting multiple sclerosis (RRMS) patients were randomly assigned to either interferon-[beta]-1a 8 MIU subcutaneously on alternate days or intramuscular interferon-[beta]-1a 30 μg once weekly. Patients and evaluating physicians were aware of the treatment assignment. At 2 years, there was an apparent treatment advantage (more patients were relapse free and there were fewer new T2 MRI lesions) in the interferon-[beta]-1b group. The treatment benefit appeared to increase in year 2, and secondary outcome measures also favored the interferon-[beta]-1b group. The relatively prolonged period of follow up (as compared with that in EVIDENCE, discussed below) is a strength of the study but, as mentioned above, the lack of evaluator and patient

blinding is an important weakness.

Trial (number enrolled)	Therapeutic agent and regimens	Follow up	Outcomes: Primary	Secondary	Comments
INCOMIN (188) [9••]	30 μg IFN-β-1a i.m. once weekly versus 9 MIU IFN-β-1b s.c. every 2 days	2 years	Proportion relapse free (61.5% versus 34.5%) and proportion free of new MRI lesions (23% versus 26%) favour IFN-β-1b	IFN-β-1b superior in terms new lesions, Gd+ lesions, and MRI activity	Second year benefit extends benefit at 1 year, only radiologist blinded
EVIDENCE (677) [8••]	IFN-β-1a 30 μg i.m. once weekly, versus 44 μg s.c. three weekly	6 months	Proportion relapse free at 6 months (74.9% versus 53.3%) and number of active lesions/patient/scan (P = 0.001)	9 month relapse rate 0.29 versus 0.40 mean active MRI lesions/patient/scan (P = 0.001)	Efforts to blind the evaluators but not patients; high dropout rate increased sensitivity at this short trial; higher rate of injection site reactions, and abnormal laboratory tests (LFTs and LCs) and NABs in s.c. group; meaning of short study; meaning of high NAB titers
European IFN-β-1a Dose Comparison Study (802) [9•]	IFN-β-1a, 30 μg i.m. once weekly versus 60 μg i.m. once weekly	36 months	No difference in EDSS progression at 1.0 to 6 months	No secondary measures significantly different	NABs low higher in 60 μg group (2.0% versus 0.8%)

EDSS, Expanded Disability Status Scale; EVIDENCE (EVidence of Interferon Dose-response: European North American Comparative Efficacy); Gd+, gadolinium enhancing MRI lesions; INCOMIN, Independent Comparison of Interferon; IFN, interferon; i.m., intramuscular; LCs, leukocyte counts; LFTs, liver function tests; MRI, magnetic resonance imaging; NAB, neutralizing antibody; s.c., subcutaneous.

Table 1. Randomized trials in relapsing-remitting multiple sclerosis: 2002

In the EVIDENCE trial [8••], 677 RRMS patients were randomly assigned to either interferon-[beta]-1a 44 μg subcutaneously three times weekly or interferon-[beta]-1a 30 μg intramuscularly once weekly. Clinical and MRI evaluators (but not patients) were blinded to the treatment received. At 6 months the patients receiving subcutaneous interferon-[beta]-1a were more likely to be relapse free and to have fewer active MRI lesions than were those receiving intramuscular interferon-[beta]-1a. The three times weekly subcutaneous group was much more likely to develop NABs than was the group receiving intramuscular treatment once weekly, however (25% versus 2%). Continued benefit was reported at 52 weeks but the difference between the two treatment groups was less apparent.

The INCOMIN and EVIDENCE studies have polarized the MS community [10,11]. The INCOMIN data add some support to previous studies indicating that there may be merit in prescribing a higher dose of IFN [1,12,13]. Others argue that the lack of evaluator blinding may have exaggerated any difference in relapse rates between the treatment groups. The EVIDENCE study is the shortest 'definitive' RCT published at the time of this review, and the high rate of early NAB formation raises concern that the early treatment response may diminish within months. These two points were not lost on those defending the 'prolonged' benefit seen in earlier studies with once weekly dosing, although it merits repeating that the follow up of patients in the original pivotal trial of interferon-[beta]-1a 30 μg intramuscularly once weekly was relatively limited as well (78%, 57%, and 10% of enrolled patients were seen at follow up at 18, 24, and 36 months, respectively) [2]. Clearly neither study advances our knowledge of whether treatment confers a prolonged benefit. As demonstrated by a recently published prospective cohort study [14••], reversible worsening ('transient treatment failure') is common, particularly in RRMS patients with mild disability, and therefore more protracted periods of follow up are needed to determine whether there is a durable benefit from treatment.

The European interferon-[beta]-1a dose comparison study (Table 1) [9•] demonstrated no apparent additional benefit from doubling the amount of drug administered by intramuscular injection once weekly (from 30 to 60 μg). Although the sponsors of that study may interpret these results to mean that the original 30 μg dose is optimal, others may speculate that more frequent administration may be needed to achieve maximal efficacy.

A small, open label, three-arm trial demonstrated an apparently comparable affect on relapse rate reduction between interferon-[beta]-1a intramuscular once weekly and either daily or alternate day subcutaneously injected GA [15]. A trend toward less adequate control of disability progression was noted in the GA-treated patients. That study exemplifies the methodologic concerns outlined above, however, being both unblinded and too small to demonstrate meaningful differences between groups.

A further analysis of the Controlled High-Risk Subjects Avonex Multiple Sclerosis Prevention Study (CHAMPS) [16] demonstrated that conversion of placebo-treated patients to clinically definite MS at 18 months was partly predicted by the degree of MRI abnormality at baseline. In a second report, Beck *et al.* [17••] reported that the originally described initial delay in clinical relapse resulting from interferon-[beta]-1a treatment extends to patients with each of three initial clinical presentations of inflammatory/demyelinating disease (optic neuritis, or brain stem/cerebellar or cord disorders).

Ovid: Noseworthy: Curr Opin Neurol, Volume 16(3),June 2003,289-297 http://gateway1.ovid.com:80/ovidweb.cgi

Of some interest, however, is the finding that within 24 months after starting treatment at least 50% of interferon-[beta]-1a-treated patients had developed evidence of a 'combined clinically definite/MRI outcome' (either clinically definite MS or at least one new or enlarging T2 lesion on scans done at 6, 12, or 18 months). Interferon-[beta]-1a administration, therefore, provides partial benefit in suppressing evidence of disease activity for at least 2 years, but many treated patients continue to have active disease. Longer trials will be needed to determine whether early treatment alters the long-term clinical course to the degree needed to delay late clinical progression.

The issue of NAB formation following interferon treatment continues to receive attention, although there remain no definitive data to guide on how to reduce NAB formation, how to reduce the levels once they have developed, or, indeed, what their clinical relevance is [18-20]. In a randomized trial of 161 RRMS patients [21], intravenous methylprednisolone (1 g, repeated monthly for 1 year) was demonstrated to reduce NAB formation in response to interferon-[beta]-1b administration to about 50% of that in the control group. Further studies are anticipated.

Continued MRI benefit (as compared with placebo) was reported in the second 9 months following treatment with GA in the extension phase of the European/Canadian MRI trial [22•], and a second, small trial demonstrated benefit from GA on development of new MRI lesions [23•]. The oral GA trial in RRMS was found to be negative (no benefit) although the results have not been published to date.

In a multi-arm, multiple dose, phase 2 trial (47 treated, 12 placebo) of 6 months' duration [24], the recombinant humanized immunoglobulin G_1 monoclonal antibody ATM-027 was shown to deplete V[beta] 5.2/5.3$^+$ T cells, reduce the expression of T-cell receptors, and reduce T-cell reactivity to some myelin antigens. This treatment was without apparent toxicity. Serial studies showed a minor degree of reduced MRI activity (perhaps 10-30%), but not to a level of statistical significance. Prolonged (18 months) V[beta] 5.2/5.3$^+$ T-cell depletion was observed in a second, small study (14 patients) [25].

The results of an open pilot study of estradiol in MS [26] and a report describing the immunomodulating effects of cholesterol-lowering statins [27••] have fueled support for definitive large-scale MS trials [28].

A Cochrane Database systematic review [29] concluded that methylprednisolone is beneficial for short-term management of acute exacerbations in MS, but there remain no data on either preventing future attacks or long-term benefit. One recent study found that the Multiple Sclerosis Functional Composite (MSFC) [30] might be more sensitive to change than the Expanded Disability Status Scale (EDSS) in detecting functional improvement in the setting of an acute relapse [31].

Progressive multiple sclerosis

The situation remains equally unclear for the management of patients with progressive forms of MS, although 2002 saw a number of studies come to completion.

In the International MS Secondary Progressive Avonex Controlled Trial (IMPACT) study (Table 2) [32••], 436 secondary progressive multiple sclerosis (SPMS) patients were randomly assigned to either interferon-[beta]-1a 60 μg intramuscularly once weekly or placebo. The primary outcome of a favorable benefit on the MSFC was realized in the second year of the study. The degree of effect of interferon-[beta]-1a on disease course and the response of the US Food and Drug Administration to these findings remain to be determined, however. Proponents point to the sensitivity of the MSFC in identifying clinical differences that are not measured well by the EDSS [33] (e.g. upper limb and cognitive function), and yet this outcome measure has not been validated or considered by the Food and Drug Administration to be an accepted primary outcome measure. Currently, there is little familiarity among the MS research community with the MSFC. There remains uncertainty about the meaning of a change of Z scores. Certainly, the failure of the trial to demonstrate a favorable effect on the usual standard outcome (EDSS) lessens the likelihood that physicians will be confident that interferon-[beta]-1a will provide a clinically obvious slowing in disease progression in SPMS patients, particularly those who are slowly worsening without clinical relapses.

Trial (number enrolled)	Therapeutic agent and regimens	Follow up	Outcomes: Primary	Outcomes: Secondary	Outcomes: Questions
IMPACT (436) [32••]	IFNβ-1a 60 μg i.m. once weekly versus placebo	2 years	Benefit on MSFC; reap1 in year 2	No effect on EDSS	MSFC not validated as a disability measure; MRI atrophy measure not reported
European mitoxantrone [33••] (188; worsening RRMS and SPMS)	Mitoxantrone 5 or 12 mg/m² every 12 weeks versus placebo (methylene blue)	2 years	Benefit on composite measure (EDSS, 9-HPT, SNS, time to first assist needing steroids, time to attack)	Number of patients with EDSS progression; MRI effects (subset of 110 patients)	Two of 5 outcome measures are coprimeasized and the evaluators of relapses was not blinded; outcome measure not validated; small numbers - not yet replicated; questionably
Belgian mitoxantrone [43•] [34]	10 treatments at 3 months: 12 mg/m² mitoxantrone versus 1 g methylprednisolone	32 months (24/45 completed)	EDSS at 36 months (not analyzed because of high dropout rate)	Improvements noted in early follow-up: 1-year EDSS improved (P < 0.0022), total number of relapses, mean number of relapses/patient/year; number of Gd⁺ enhancing lesions improved	50% failed to complete the trial; no intent-to-treat analysis

Table 2. Randomized trials in secondary progressive multiple sclerosis: 2002

Baseline variables and results	Placebo: EUSPMS [37]	Placebo: SPEC-TRIMS [13]	Placebo: NASPMS [36]	Placebo: IMPACT [32••]	Active treatment groups: EUSPMS [37] 6 MIU every other day	SPECTRIMS [13]: 22 mg thrice weekly	SPECTRIMS [13]: 44 μg thrice weekly	NASPMS [36]: 9 MIU every other day	NASPMS [36]: 9 MIU/m² every other day	IMPACT [32••]: 80 μg every week i.m.
n	308	204	308	218	260	209	253	317	314	217
Baseline										
% Female	64	60	60	64	58	62	67	66	61	61
Mean age (years)	45.9	42.7	47.6	47.9	41.1	43.1	42.6	46.1	46.9	47.2
Mean EDSS	6.2	5.4	6.1	6.2	6.1	6.6	6.4	6.2	6.1	6.2
Disease duration (since diagnosis; years)	13.4	13.7	11.9	10.8	12.8	13.3	12.9	11.6	11.8	10.2
Duration of SP (since onset)	3.8	4.1	4.1	NA	3.8	4.2	3.7	4.0	4.0	NA
Enrolled/eligible (%)	29.2	62	56	44/80[a]	31.9	64	51	64	55	37/59[a]
Results										
Lost to follow-up (%)	6.2	0.3	10.4	10.6	7.2	7.7	6.9	13.9	8.9	13.3
Overall treatment (%)	27.1	10.6	24.4	24.7	23.0	17.7	21.1	24.9	23.9	20.1
Blinding (patients, %)[b]	64	62	36	NA	56	59	59	51%	56	NA
Blinding (EDSS-physicians, %)[c]	16	20	9	NA	21	29	29	7%	8	NA
Confirmed progression (%)[d]	64	65	37	27	45[e]	60	59	37	44	26
Exacerbation free (%)	36	37	62	63	43[f]	42[g]	44[h]	21[i]	52[g]	74[j]
Median days to first relapse	285	291	NR	NR	644	476	494	NR	NR	NR
No MRI active, new and enlarging T2 lesions, (%)	16	24	31	42	36[k]	36[k]	41[k]	50[l]	44[l]	62[k]
Median change in T2 volume (%)	6.7	10.0	10.9	NA	5.3[m]	0.0[l]	1.0[l]	0.1[l]	NA	NA

[a]Values for previous 2/1 years (% relapse free in the last 5 years and one year, respectively); [b]Correct guesses regarding assigned treatment (%); [c]Confirmed at 3 months: EUSPMS and SPECTRIMS, combined at 6 months: NASPMS and IMPACT; [d]P < 0.05–0.001. EDSS, Expanded Disability Status Scale; EUSPMS, European secondary progressive MS interferon β-1b trial; IFN, interferon; i.m., intramuscular; IMPACT, International MS Secondary Progressive Avonex Controlled Trial; MRI, magnetic resonance imaging; NA, not available; NASPMS, North American Secondary Progressive MS Trial; NR, not reached; SP, secondary progressive; SPECTRIMS, Secondary Progressive Efficacy Clinical Trial of Recombinant Interferon β-1a in MS trial. Data from Kappos [38]

Table 3. Summary of major differences in the secondary progressive multiple sclerosis interferon trials

The full report of the North American Interferon-[beta]-1b study in SPMS has not been published, although results presented in abstract form were disappointing [36]. As such, it remains somewhat unclear why the European Interferon-[beta]-1b in SPMS study (EUSP) [37] was 'positive' in the face of a failure of other studies [e.g. Secondary Progressive Efficacy Clinical Trial of Recombinant Interferon [beta]-1a in MS trial (SPECTRIMS) [13], International MS Secondary Progressive Avonex Controlled Trial (IMPACT) [32••] and the North American study [36]] to demonstrate benefit in the development of disability as measured using the EDSS (even the 'positive IMPACT' study did not demonstrate a beneficial change in EDSS). It appears likely that important differences in clinical baseline characteristics between the studies will offer a partial explanation (Table 3). Essentially, the patients in the first completed trial (EUSP) were more likely to have a shorter duration of disease progression and a greater likelihood of having recurring relapses than were patients in the subsequent SPMS studies. Each of the four large studies demonstrated that the [beta] interferons continue to reduce clinical and MRI markers of inflammatory activity, even during the secondary progressive phase of the illness. Regrettably, the clinical (disability progression) and late MRI counterparts of axonal degeneration (e.g. atrophy [39]) are less clearly altered by interferon treatment started during the secondary progressive phase of the illness.

A study from Belgium (Table 2) demonstrated that relapses and MRI evidence of disease activity were more likely to be favorably influenced by mitoxantrone than by methylprednisolone during the first year after starting treatment [34]. That trial was designed to measure a 3-year benefit from treatment, but fully 50% dropped out before this end-point was reached. The long-awaited report of the Mitoxantrone in Multiple Sclerosis study group has now been published [33••]. In that phase 3, double-blinded study, 194 patients with either worsening RRMS or SPMS were randomly assigned to receive either placebo or one of two doses of mitoxantrone every 3 months for 2 years (Table 2). MRI measures were evaluated in a subset of 110 patients. The primary outcome (a combined multivariate outcome of five clinical measures of worsening), most of the secondary clinical outcome measures, and preliminary MRI evidence suggested a treatment benefit for the higher dose (12 mg/m^2), although one outcome (the presence and severity of relapses) was evaluated by an unblinded treating physician. Only 149 patients finished 2 years of the study (76%), however, with substantial numbers of dropouts in all three treatment arms (18 placebo, and 27 in each of the two active treatment arms). These findings, together with the results of a previous study [40], led to US Food and Drug Administration approval of this agent for active forms of relapsing MS and SPMS in 2000, although the data were only reported in full form in December 2002. Additional studies are now needed to verify that report; clarify whether there is a benefit regardless of the presence of ongoing clinical relapses in SPMS patients; determine the optimal patient selection criteria and dosing schedules; benefits of combining treatment with interferons or GA; and the long-term safety of this anthrecenedione. Secondary amenorrhea lasting at least 1 year (5 out of 25 patients in the 12 mg/m^2 group), nausea, leukopenia, possibly an increased risk for leukemia, and perhaps most importantly a dose-dependent cardiomyopathy [41] are important adverse effects of this agent. Whether mitoxantrone is truly effective in delaying late progression or is preferable to other cytotoxic agents (e.g. cladribine, cyclophosphamide, azathioprine) remains unknown.

Although not yet published, disappointing results of large trials of intravenous immunoglobulin in SPMS and GA in primary progressive MS were announced in the final quarter of 2002.

Adverse treatment effects

In an open label study, paracetamol and acetominophen [42] were shown to be effective in controlling interferon-[beta]-1a related flu symptoms. Isolated case reports added cutaneous mucinoses [43•] and panniculitis [44•] to the list of occasional complications of subcutaneous injection of interferon-[beta]-1b. The first report of rhabdomyolysis [45•] and another severe case of autoimmune hepatitis responsive to corticosteroids and azathioprine

[46•] were reported as rare complications of subcutaneous and intramuscular interferon-[beta]-1a administration, respectively. A 1-year prospective study of 42 RRMS patients [47] suggested that interferon-[beta]-1b does not significantly increase the risk for depression and that a previous history of depression (but not a family history, alone) may predispose to depression following initiation of interferon-[beta]-1b. The authors of that report concluded that interferon-[beta]-1b can often be continued and that antidepressant medications are helpful when depression develops in this setting. In a combined prospective and retrospective report [48], investigators concluded that interferon-[beta] is more likely than GA to be associated with troublesome headaches, although the conclusions about GA were derived from the retrospective data only.

Sympt matic therapies

A Cochrane Database systematic review [49] failed to reach a definitive conclusion about the possible benefit of the 4-, and 3,4-aminopyrindines. The authors noted that published data suggested some benefit, particularly in motor function and ambulation, without excessive toxicity (encephalopathy and seizures occur infrequently), but they noted the possible role of publication bias in that three RCTs (>300 patients) remained unpublished. Modafanil was felt to be both safe and modestly effective in treating MS fatigue in one short, single-blinded, placebo-controlled study of 72 patients [50]. A double-blinded study is needed [51]. Neither oral [DELTA]^9-tetrahydrocannabinal nor *Cannabis sativa* plant extract reduced spasticity, and both worsened MS patients' subjective global impression in a randomized, small, double-blind, placebo-controlled, crossover study [52].

Future options

To date there is no consensus on what constitutes 'responder' and 'nonresponder' status. The Association of British Neurologists created guidelines for discontinuing treatment, but no recommendations have reached widespread acceptance [53•]. It is important to realize that the demographics of the 'responders' have not been adequately characterized or validated prospectively, despite nearly a decade of approved therapies.

The hypothesis that there may be a limited (e.g. four or less) number of patterns of tissue injury that may persist within individuals for the duration of their illness raises the promise that it may be possible to target specific therapies to individual patient groups with better success than has been done to date [54]. There are now early observations that neuromyelitis optica [55] and some MS variants may be mediated by humoral mechanisms, and are therefore responsive to plasma exchange or immunoglobulin administration [56-59,60••].

Conclusion

RCT methodology has been used to great advantage to identify differences between active agents and placebo in short-term studies. As noted above, there has recently been a move toward shorter trials with less care given to the elements of trial design and conduct that are classically thought to be essential (e.g. adequate patient and evaluator blinding, and the need for intent-to-treat analyses). The MS trial field may now be at a true crossroads: continue to apply RCT methodology to the large variety of candidate MS treatment strategies (a partial listing is shown in Table 4) and yet abandon, if convenient, critical quality design elements in order to meet the desired purpose of the sponsor (e.g. do the shortest trial possible to demonstrate an early benefit); or create an alternative strategy to identify the size and duration of putative therapeutic effectiveness. The key question facing the clinical trial field may concern the extent to which early, immediate (weeks) down-regulation of inflammatory/demyelinating damage to the target tissue (oligodendrocyte, myelin sheath, or axon) may influence the later course of this chronic illness. There appear to be only a few options available for such a 'paradigm shift'. These options include large-scale prolonged RCTs, expanded registries, and observational cohort studies [61]. If statistical modeling techniques can be applied successfully to MS (perhaps using the resources of the Sylvia Lawry Centre for MS Research [62] or other large databases), then it may ultimately be possible to recognize unequivocal differences in treatment protocols over prolonged periods of observation using an alternate approach to the current RCT methodology.

Target	Strategy
Blood-brain barrier	Anti-adhesion molecule mAbs (anti-VLA-4 mAb) Anti-α4 integrin antibody ± IFN-β-1a Matrix metalloproteinase inhibitors Tat/an inhibitor of matrix metalloproteinase promoters Chemokine blockers, corticosteroids Anoxia Nitric oxide synthase inhibitor
Class II	Anti-MHC mAbs Hypervariable peptide vaccines Blockers of IFN-γ, interleukin-12, osteopontin
Antigen	Altered peptide ligands Antigen/MHC complex binders
TCR	TCR peptides TCR vaccines Anti-TCR mAbs
Costimulatory T-cell ligands	Anti-CD40 ligand Anti-B7 mAbs CTLA-4 immunoglobulin fusion protein CD40-CD40 ligand interaction
Th1 cell	Interleukin-1 inhibitors Interleukin-1 receptor blockade Anti-interleukin-12 Campath-1H (anti-CD52) Anti-interleukin-2 receptor α
Th2 cell	Interleukin-10 Estriol
Cell recruitment	Anticytokines, chemokines Theofemide, pentoxifylline
Macrophage, astrocyte	Inhibitors of activation products (arachidonic acid, platelet-activating factor, free radicals, glutamate, quinolinic, cysteine, amines, chemokines) Cytokines (tumor necrosis factor-α, interleukin-1β) Protein kinase inhibitors Caspase signaling inhibitors Nonimmunophilins
Anti-CD8 cell	Chemotherapy Anti-CD8+ mAbs
Oligodendrocyte protection	Neuroprotection strategies
Complement	Anticomplement mAbs Inhibitors of complement membrane attack complex interactions
Demyelinating antibodies	Plasma exchange IVIg Immunosuppressants Monoclonal anti-idiotypic antibodies Anti-B cell mAbs
Remyelinating antibodies	IVIg Humanized natural immunoglobulin M mAbs
Transplantation	Oligodendrocytes (fetal, adult) Schwann cells Cell lines Stem cells, progenitors Olfactory ensheathing glia Xenogeneic cells
Growth factors	Insulin-like growth factor-1, platelet-derived growth factor, ciliary neurotrophic growth factor, neurotrophin-3 Interleukin-6 Gene therapy
Neuroprotection	Excitotoxic/oxidative stress (channel blockers (Na^+, Ca^{++}), glutamate inhibition, γ-aminobutyric acid stimulation) Nitric oxide synthase pathway downregulation Antiapoptosis strategies
Axon regeneration	Axon guidance (remyelinating neuregulin, glycan) Therapeutic antibodies (IGF-1 antibody, anti-Nogo antibodies) Protective factors: nerve growth factor, neurotrophin-3, brain-derived growth factor, basic fibroblast growth factor, interleukin-10

CTLA, cytotoxic T-lymphocyte antigen; IFN, interferon; IVIg, intravenous immunoglobulin; MAb, monoclonal antibody; MHC, major histocompatibility complex; TCR, T-cell receptor; Th, T-helper; VLA, very late antigen. Adapted from Wingerchuk et al. [5].

Table 4. Possible future multiple sclerosis treatment strategies

There is hope that increasingly sophisticated scientific methods (including gene expression studies and proteomics) may soon help to unravel the most important mechanisms of tissue injury and response to therapy in MS, and thereby guide the search for meaningful therapies [63,64,65•].

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

•of special interest

••of outstanding interest

1 The IFNB Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group. Interferon [beta]-1b in the treatment of MS: final outcome of the randomized controlled trial. Neurology 1995; 45:1277-1285. [Context Link]

2 Jacobs LD, Cookfair DL, Rudick RA, *et al*. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). Ann Neurol 1996; 39:285-294.

[BIOSIS Previews Link] [Context Link]

3 Johnson KP, Brooks BR, Cohen JA, *et al*. Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group. Neurology 1995; 45:1268-1276. [BIOSIS Previews Link] [Context Link]

4• Wiendl H, Hohlfeld R. Therapeutic approaches in multiple sclerosis: lessons from failed and interrupted treatment trials. Biodrugs 2002; 16:183-200. This is a balanced, thoughtful and thorough review of recent MS trials, many of which are disappointing but informative. [Context Link]

5 Wingerchuk D, Lucchinetti C, Noseworthy J. Multiple sclerosis: current pathophysiological concepts. Lab Invest 2001; 81:263-281. [BIOSIS Previews Link] [Context Link]

6 Rice GP, Incorvaia B, Munari L, *et al*. Interferon in relapsing-remitting multiple sclerosis [Cochrane Systematic Review]. Oxford: Update Software; 2002. [Context Link]

7•• Durelli L, Verdun E, Barbero P, *et al*. Every-other-day interferon beta-1b versus once-weekly interferon beta-1a for multiple sclerosis: results of a 2-year prospective randomised multicentre study (INCOMIN). Lancet 2002; 359:1453-1460. See [8••]. [Context Link]

8•• Panitch H, Goodin DS, Francis G, *et al*. Randomized, comparative study of interferon beta-1a treatment regimens in MS: the EVIDENCE Trial. Neurology 2002; 59:1496-1506. [BIOSIS Previews Link] This trial and that by Durelli *et al*. [7••], along with the accompanying editorials [10,11], highlight the complexities of head-to-head comparison trials of active agents with relatively brief periods of follow up. This will be a recurring area of interest in MS therapeutics. [Context Link]

9• Clanet M, Radue EW, Kappos L, *et al*. A randomized, double-blind, dose-comparison study of weekly interferon beta-1a in relapsing MS. Neurology 2002; 59:1507-1517. [BIOSIS Previews Link] This study suggests that there is no advantage to increasing the once weekly intramuscular dose of interferon-[beta]-1a beyond 30 μg. [Context Link]

10 Lublin FD. When marketing and science intersect: do patients with MS benefit? Neurology 2002; 59:1480-1481. [Context Link]

11 Kieburtz K, McDermott M. Needed in MS: evidence, not EVIDENCE. Neurology 2002; 59:1482-1483. [Context Link]

12 Prisms Study Group, University of British Columbia MS MRI Analysis Group. PRISMS-4: long-term efficacy of interferon-beta-1a in relapsing MS. Neurology 2001; 56:1628-1636. [BIOSIS Previews Link] [Context Link]

13 Secondary Progressive Efficacy Clinical Trial of Recombinant Interferon-beta-1a in MS Study Group. Randomized controlled trial of interferon-beta-1a in secondary progressive MS: clinical results. Neurology 2001; 56:1496-1504. [BIOSIS Previews Link] [Context Link]

14•• Rio J, Nos C, Tintore M, *et al*. Assessment of different treatment failure criteria in a cohort of relapsing-remitting multiple sclerosis patients treated with interferon beta: implications for clinical trials. Ann Neurol 2002; 52:400-406. [BIOSIS Previews Link] This report clearly shows that many patients (especially those with mild clinical disability) who worsen on treatment eventually recover to a significant degree, as measured by disability scales. This study thereby demonstrates the importance of prolonged follow up in determining the stability of early clinical deterioration in the setting of clinical trials (and practice). [Context Link]

15 Flechter S, Vardi J, Pollak L, Rabey JM. Comparison of glatiramer acetate (Copaxone) and interferon beta-1b (Betaferon) in multiple sclerosis patients: an open-label 2-year follow-up. J Neurol Sci 2002; 197:51-55. [BIOSIS Previews Link] [Context Link]

16 Champs Study Group. MRI predictors of early conversion to clinically definite MS in the CHAMPS placebo group. Neurology 2002; 59:998-1005. [BIOSIS Previews Link] [Context Link]

17•• Beck RW, Chandler DL, Cole SR, *et al*. Interferon beta-1a for early multiple sclerosis: CHAMPS trial subgroup analyses. Ann Neurol 2002; 51:481-490. [BIOSIS Previews Link] This study provides additional detail about the magnitude of treatment response in the CHAMPS trial. Many patients (approximately 50%) will demonstrate evidence of continued clinical or MRI activity while on treatment with interferon-[beta]-1a within the first 2 years of initiating therapy. [Context Link]

18 Scagnolari C, Bellomi F, Turriziani O, *et al*. Neutralizing and binding antibodies to IFN-beta: relative frequency in relapsing-remitting multiple sclerosis patients treated with different IFN-beta preparations. J Interferon Cytokine Res 2002; 22:207-213. [BIOSIS Previews Link] [Context Link]

19 Bertolotto A, Malucchi S, Sala A, *et al*. Differential effects of three interferon betas on neutralizing antibodies in patients with multiple sclerosis: a follow up study in an independent laboratory. J Neurol Neurosurg Psychiatry 2002; 73:148-153. [BIOSIS Previews Link] [Context Link]

20 Coles AJ. Neutralising antibodies to the beta interferons. J Neurol Neurosurg Psychiatry 2002; 73:110-111. [BIOSIS Previews Link] [Context Link]

21 Pozzilli C, Antonini G, Bagnato F, *et al*. Monthly corticosteroids decrease neutralizing antibodies to IFN beta1 b: a randomized trial in multiple sclerosis. J Neurol 2002; 249:50-56. [BIOSIS Previews Link] [Context Link]

22• Wolinsky JS, Comi G, Filippi M, *et al*. Copaxone's effect on MRI-monitored disease in relapsing MS is reproducible and sustained. Neurology 2002; 59:1284-1286. [BIOSIS Previews Link] See [23•]. [Context Link]

23• Rovaris M, Codella M, Moiola L, *et al*. Effect of glatiramer acetate on MS lesions enhancing at different gadolinium doses. Neurology 2002; 59:1429-1432. [BIOSIS Previews Link] This study and that by Wolinsky *et al*. [22•] provide supportive evidence that GA favorably influences MRI activity in RRMS. [Context Link]

24 Killestein J, Olsson T, Wallstrom E, *et al*. Antibody-mediated suppression of Vbeta5.2/5.3$^+$ T cells in multiple sclerosis: results from an MRI-monitored phase II clinical trial. Ann Neurol 2002; 51:467-474. [BIOSIS Previews Link] [Context Link]

25 Olsson T, Edenius C, Ferm M, *et al*. Depletion of Vbeta5.2/5.3 T cells with a humanized antibody in patients with multiple sclerosis. Eur J Neurol 2002; 9:153-164. [BIOSIS Previews Link] [Context Link]

26 Sicotte NL, Liva SM, Klutch R, *et al*. Treatment of multiple sclerosis with the pregnancy hormone estriol. Ann Neurol 2002; 52:421-428. [BIOSIS Previews Link] [Context Link]

27•• Neuhaus O, Strasser-Fuchs S, Fazekas F, *et al*. Statins as immunomodulators: comparison with interferon-beta 1b in MS. Neurology 2002; 59:990-997. [BIOSIS Previews Link] These two studies have stimulated considerable attention for future trials of estrogens and statins in MS. [Context Link]

28 Zamvil SS, Steinman L. Cholesterol-lowering statins possess anti-inflammatory activity that might be useful for treatment of MS. Neurology 2002; 59:970-971. [BIOSIS Previews Link] [Context Link]

29 Filippini G, Brusaferri F, Sibley WA, *et al*. Corticosteroids or ACTH for acute exacerbations in multiple sclerosis [Cochrane Systematic Review]. Oxford: Update Software; 2002. [Context Link]

30 Cutter GR, Baier ML, Rudick RA, *et al*. Development of a multiple sclerosis functional composite as a clinical trial outcome measure. Brain 1999; 122:871-882. [BIOSIS Previews Link] [Context Link]

31 Patzold T, Schwengelbeck M, Ossege LM, *et al*. Changes of the MS functional composite and EDSS during and after treatment of relapses with methylprednisolone in patients with multiple sclerosis. Acta Neurol Scand 2002; 105:164-168. [BIOSIS Previews Link] [Context Link]

32•• Cohen JA, Cutter GR, Fischer JS, *et al*. Benefit of interferon beta-1a on MSFC progression in secondary progressive MS. Neurology 2002; 59:679-687. [BIOSIS Previews Link] This study again shows that interferon-[beta]-1a is partially active against clinical and MRI evidence of ongoing inflammatory pathology in MS (relapses and MRI activity). A new, presumably more sensitive outcome measure (the MSFC measure) showed evidence for slowing of disability progression. Once again, however, there was no evidence of benefit from this treatment using the traditional, standard measure of change in disability (Kurtzke's EDSS). [Context Link]

33•• Hartung H-P, Gonsette R, Konig N, *et al*. Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, muticentre trial. Lancet 2002; 360:2018-2025. This trial and that reported by van de Wyngaert *et al*. [34] (together with the report by Edan *et al*. [40]) provide additional evidence that mitoxantrone reduces active inflammatory pathology in active and progressive forms of MS. Additional studies to extend these early findings and clarify indications, dosing, and safety issues are clearly needed. [Context Link]

34 van de Wyngaert FA, Beguin C, D'Hooghe MB, *et al*. A double-blind clinical trial of mitoxantrone versus methylprednisolone in relapsing, secondary progressive multiple sclerosis. Acta Neurol Belg 2001; 101:210-216.

[Context Link]

35 Kurtzke JF. Rating neurological impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 1983; 33:1444-1452. [BIOSIS Previews Link] [Context Link]

36 Goodkin DE, the North American Study Group on Interferon beta-1b in Secondary Progressive MS. Interferon beta-1b in secondary progressive MS: clinical and MRI results of a 3-year randomized controlled trial [abstract]. Neurology 2000; 54:2352. [Context Link]

37 Kappos L, Polman C, Pozzilli C, *et al*. Final analysis of the European multicenter trial on IFNb-1b in secondary-progressive MS. Neurology 2001; 57:1969-1975. [BIOSIS Previews Link] [Context Link]

38 Kappos L. Interferons. In: Rudick R, Cohen J, editors. Multiple sclerosis therapeutics. London: Martin Dunitz; 2003 (in press)

39 Fisher E, Rudick RA, Simon JH, *et al*. Eight-year follow-up study of brain atrophy in patients with MS. Neurology 2002; 59:1412-1420. [BIOSIS Previews Link] [Context Link]

40 Edan G, Miller D, Clanet M, *et al*. Therapeutic effect of mitoxantrone combined with methylprednisolone in multiple sclerosis: a randomized multicenter study of active disease using MRI and clinical criteria. J Neurol 1997; 62:112-118. [Context Link]

41 Strotmann JM, Spindler M, Weilbach FX, *et al*. Myocardial function in patients with multiple sclerosis treated with low-dose mitoxantrone. Am J Cardiol 2002; 89:1222-1225. [BIOSIS Previews Link] [Context Link]

42 Reess J, Haas J, Gabriel K, *et al*. Both paracetamol and ibuprofen are equally effective in managing flu-like symptoms in relapsing-remitting multiple sclerosis patients during interferon beta-1a (AVONEX) therapy. Mult Scler 2002; 8:15-18. [BIOSIS Previews Link] [Context Link]

43• Benito-Leon J, Borbujo J, Cortes L. Cutaneous mucinoses complicating interferon beta-1b therapy. Eur Neurol 2002; 47:123-124. [BIOSIS Previews Link] See [46•]. [Context Link]

44• Heinzerling L, Dummer R, Burg G, Schmid-Grendelmeier P. Panniculitis after subcutaneous injection of interferon beta in a multiple sclerosis patient. Eur J Dermatol 2002; 12:194-197. See [46•]. [Context Link]

45• Lunemann JD, Schwarzenberger B, Kassim N, *et al*. Rhabdomyolysis during interferon-beta 1a treatment. J Neurol Neurosurg Psychiatry 2002; 72:274. (Erratum appears in J Neurol Neurosurg Psychiatry 2002; 73:354.) See [46•]. [Context Link]

46• Duchini A. Autoimmune hepatitis and interferon beta-1a for multiple sclerosis. Am J Gastroenterol 2002; 97:767-768. [BIOSIS Previews Link] Clinicians are reminded by this report and [43•-45•] to be cautious in evaluating systemic illnesses that occur during treatment with approved MS therapies. [Context Link]

47 Feinstein A, O'Connor P, Feinstein K. Multiple sclerosis, interferon beta-1b and depression. A prospective investigation. J Neurol 2002; 249:815-820. [BIOSIS Previews Link] [Context Link]

48 Pollmann W, Erasmus LP, Feneberg W, *et al*. Interferon beta but not glatiramer acetate therapy aggravates headaches in MS. Neurology 2002; 59:636-639. [BIOSIS Previews Link] [Context Link]

49 Solari A, Uitdehaag B, Giuliani G, *et al*. Aminopyridines for symptomatic treatment in multiple sclerosis [Cochrane Systematic Review]. Oxford: Update Software; 2002. [Context Link]

50 Rammohan KW, Rosenberg JH, Lynn DJ, *et al*. Efficacy and safety of modafinil (Provigil) for the treatment of fatigue in multiple sclerosis: a two centre phase 2 study. J Neurol Neurosurg Psychiatry 2002; 72:179-183. [BIOSIS Previews Link] [Context Link]

51 Willoughby E. Modafinil for fatigue in multiple sclerosis [editorial]. J Neurol Neurosurg Psychiatry 2002; 72:150. [BIOSIS Previews Link] [Context Link]

52 Killestein J, Hoogervorst EL, Reif M, *et al*. Safety, tolerability, and efficacy of orally administered cannabinoids in MS. Neurology. 2002; 58:1404-1407. [BIOSIS Previews Link] [Context Link]

53• Little R. NHS to fund treatment for 10000 patients with MS. BMJ 2002; 324:316. This report describes one early attempt to define guidelines for stopping interferon therapy in MS patients. [Context Link]

54 Lucchinetti CF, Bruck W, Parisi J, *et al*. Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. Ann Neurol 2000; 47:707-717. [BIOSIS Previews Link] [Context Link]

55 Lucchinetti CF, Mandler RN, McGavern D, *et al*. A role for humoral mechanisms in the pathogenesis of Devic's neuromyelitis optica. Brain 2002; 125:1450-1461. [BIOSIS Previews Link] [Context Link]

56 Weinshenker B, O'Brien P, Petterson T, *et al*. A randomized trial of plasma exchange in acute CNS inflammatory demyelinating disease. Ann Neurol 1999; 46:878-886. [BIOSIS Previews Link] [Context Link]

57 Keegan M, Pineda AA, McClelland RL, *et al*. Plasma exchange for severe attacks of CNS demyelination: predictors of response. Neurology 2002; 58:143-146. [BIOSIS Previews Link] [Context Link]

58 Mao-Draayer Y, Braff S, Pendlebury W, Panitch H. Treatment of steroid-unresponsive tumefactive demyelinating disease with plasma exchange. Neurology 2002; 59:1074-1077. [BIOSIS Previews Link] [Context Link]

59 Bruck W, Neubert K, Berger T, Weber JR. Clinical, radiological, immunological and pathological findings in inflammatory CNS demyelination: possible markers for an antibody-mediated process. Mult Scler 2001; 7:173-177. [BIOSIS Previews Link] [Context Link]

60•• Gold R, Linington C. Devic's disease: bridging the gap between laboratory and clinic. Brain 2002; 125:1425-1427. [BIOSIS Previews Link] These papers (i.e. [55-59]) and this editorial by Gold and Linington provide evidence that plasma exchange may enhance recovery in subsets of MS patients. [Context Link]

61 Noseworthy JH. Use of pooled data, historical controls and long-term follow-up studies to determine safety and efficacy. In: Cohen JA, Rudick RA, editors. Multiple sclerosis therapeutics, 2nd ed. London: Martin Dunitz; 2002 (in press). [Context Link]

62 Noseworthy JH, Kappos L, Daumer M. Competing interests in multiple sclerosis research [letter]. Lancet 2003 (in press). [Context Link]

63 Martin R, Sturzebecher CS, McFarland HF. Immunotherapy of multiple sclerosis: where are we? Where should we go? Nat Immunol 2001; 2:785-788. [BIOSIS Previews Link] [Context Link]

64 Steinman L. Gene microarrays and experimental demyelinating disease: a tool to enhance serendipity. Brain 2001; 124:1897-1899. [BIOSIS Previews Link] [Context Link]

65• Tompkins SM, Miller SD. An array of possibilities for multiple sclerosis. Nat Med 2002; 8:451-453. This essay and those by Martin *et al*. [63] and Steinman [64] highlight the potential of 'the new biology' to advance the MS research field in the years ahead. [Context Link]

multiple sclerosis; randomized controlled clinical trials; clinical trial design; glatiramer acetate; beta interferon; mitoxantrone

Accession Number: 00019052-200306000-00007

Ovid: Noseworthy: Curr Opin Neurol, Volume 16(3) June 2003.289-297 http://gateway1.ovid.com:80/ovidweb.cgi

[Frontiers in Bioscience 7, d731-751, March 1, 2002]

AN INTEGRATED VIEW OF THE ROLES AND MECHANISMS OF HEAT SHOCK PROTEIN GP96-PEPTIDE COMPLEX IN ELICITING IMMUNE RESPONSE

Zihai Li, Jie Dai, Hong Zheng, Bei Liu and Marissa Caudill

Center for Immunotherapy of Cancer and Infectious Diseases, University of Connecticut School of Medicine, Farmington, CT

TABLE OF CONTENTS

1. ABSTRACT

Heat shock protein (HSP) gp96, or grp94 is an endoplasmic reticular (ER) paralog of the cytosolic HSP90. Being abundant and non-polymorphic, gp96 plays significant roles in maintaining protein homeostasis in the secretory pathway. This "house-keeping" role of gp96 has now been overshadowed by the intriguing findings that gp96 modulates both the innate and adaptive components of the immune system. It has been found that, (i) gp96 is one of the major peptide binding proteins in the ER, (ii) gp96 interacts specifically with receptors including CD91 and possibly toll-like receptors (TLRs), on the surface of professional antigen presenting cells (APCs), (iii) interaction with APCs leads to re-presentation of gp96-chaperoned peptides to the major histocompatibility complex (MHC) molecules of APCs, (iv) direct access of gp96 to APCs triggers functional activation of APCs. In this review, we will examine each of these immunological attributes of gp96 critically. As experimentalists, we will also propose specific experiments to examine the argument that gp96, perhaps along with other members of HSP family, is *the* antigenic carrier for mediating cross priming of antigen-specific T lymphocytes in vertebrates.

2. INTRODUCTION

2.1. Structurally unaltered gp96 and other HSPs are "tumor rejection antigens"

Fueled by the idea of immunosurveillance (1) and the dream that tumors can be dealt with by vaccinations, tumor immunologists have long been fascinated by the immunological differences between normal cells and their malignant counterparts. Successful tumor-specific vaccines are dependent on the proof and identification of tumor antigens, against which an immune response leads to tumor rejection. The existence of these so-called tumor rejection antigens (TRAs) was immunologically defined by a series of transplantation experiments in rodents using syngeneic, chemically-induced tumors performed as early as in the 1940s (2-6). Inactivated tumor cells were shown to immunize syngeneic animals against subsequent challenge with live tumors of the same origin. Soon it was found that this phenomenon was not restricted to tumor types or hosts. In addition, tumor immunity generated by immunization from whole tumor cells was shown to be exquisitely specific. Thus, tumor A can only immunize animals against the challenge of tumor A but not against that of tumor B, and vice versa, even if tumor A and B are derived from the same histological types, induced by the same carcinogen, or even developed in the same host. This suggests that the antigenic determinants vary among different tumors, and predicts that an effective cancer vaccine has to be derived from autologous tumors.

Since whole tumor cell lysate could also immunize and confer protection against tumors, a tedious biochemical approach was undertaken to identify the molecular basis for tumor rejection. Tumor cell lysates were fractionated biochemically. Each fraction was then injected to animals followed by live tumor challenge, to determine which fraction confers tumor protection. The active fraction was then further fractionated until a homogenous population of proteins was identified. This approach has led to the successful identification of several TRAs. Surprisingly, a majority of these TRAs turned out to be HSPs including HSP90 (7), gp96 (8-

10), HSP70 (11), calreticulin (12), HSP110 and GRP170 (13), which collectively play critical roles in facilitating protein folding and unfolding in the cell (14).

Gp96 stands for glycoprotein of 96 kDa. In humans, only one true gene locus has been mapped and was named tra-1 (15). In the literature, gp96 is also referred to as GRP94, Erp99, endoplasmin, etc (16, 17). The first report to link gp96 with tumor immunity came from Srivastava and Das who showed that in a Wistar rat Zajdela ascitic hepatoma model, a homogenous preparation of a ~100 kDa protein (named ZAH-TATA) was able to immunize against challenge with the parental tumors (8). Although ZAH-TATA was not molecularly defined in the original report, its biochemical properties suggest that ZAH-TATA is the rat gp96 homolog.

Using methylcholanthrene (MCA)-induced fibrosarcoma (Meth A) as a model in BALB/c mice, a series of papers in the 1980s described the ability of gp96 to immunize naïve mice against challenge with the tumor from which gp96 was purified (9, 10, 18). Gp96 is expressed ubiquitously in both normal and tumor cells and turned out to be identical to Erp99, an abundant protein of the endoplasmic reticulum (19), although surface expression of gp96 was also demonstrated (9, 20). Subsequently, gp96 was found to be a *bona fide* protein chaperone and HSP because of heat or stress inducibility (20, 21), adenosine nucleotide binding and ATPase activity (22), and apparent unselective binding to unfolded proteins (16). Functionally, gp96 fulfills all the criteria for a TRA. Immunization of animals with tumor-derived gp96, but not gp96 purified from normal tissues or another tumor type, protects the animals against the tumor of gp96 origin. It was immediately speculated then that there are tumor specific alterations of gp96 itself (23). Yet, surprisingly, when the cDNA of gp96 was cloned and sequenced from both tumors and normal tissues, no such mutation of gp96 gene was discovered (24, 25).

2.2. Gp96 is not antigenic per se: the hypothesis and emergence of evidence for gp96 to chaperone antigenic peptides for adaptive immunity

The protective immunity elicited by gp96 vaccination is exquisitely specific. Various possibilities that might explain this specificity were considered (23). First, it was proposed that gp96 might be an extremely polymorphic molecule, much like MHC, T cell receptor and immunoglobulin. Second, gp96 was suggested to be especially prone for somatic mutations during tumorigenesis. Thus tumor A would have a mutation pattern of gp96 distinct from that of tumor B. These two possibilities were quickly rejected thanks to molecular cloning and sequencing. Third, extensive post-translational modification and the differences generated by it could have accounted for the differences in immunogenicity of gp96. This, too, was found to be incorrect. Gp96 was expressed abundantly in both normal and tumor cells. Gp96 from different sources was found to be identical in size and charge, and has overlapping behavior on various chromatographic columns. Fourth, gp96 itself is not the true antigen; the antigenicity of gp96 preparations is actually due to trace contamination of other proteins. This argument was thought to be unlikely due to the fact that gp96 preparations were found to be homogenous by all criteria tested, including: a single band on silver stained SDS-PAGE, single-peak UV absorption curve at the last step of chromatography of standard gp96 preparation, and undetectable materials with significant mass other than a 96 kDa protein by mass spectrometry. Finally, it was postulated that gp96 preparation harbor small molecular weight peptide moieties, given the fact that tumor-specific T cells recognize tumor-derived peptides in association with MHC molecules. This hypothesis was catalyzed by the landmark work from Townsend and others at the time, which showed peptides from all cellular proteins

can be potentially charged to MHC class I molecules irrespective of their intended subcellular localization (26, 27).

This peptide-binding hypothesis, while offering an explanation for the specificity associated with gp96 vaccination, was limited by the fact that the mechanism used by gp96-peptide complexes to activate an immune response was not clear. Various hypothetical mechanisms were proposed (23,25, 28-32), many of which have now been experimentally tested, and some of which have been validated (figure 1). These hypothesis and ideas have implications for the physiological roles of gp96 in immune response.

Hypothesis one: Gp96-peptide complexes are involved in presenting peptides directly to the adaptive immune system. This would mean that gp96 is a direct antigen-presenting molecule on the cell surface, something analogous to MHC itself. This would also mean that a gp96 receptor or ligand has to be expressed on the surface of cells of the immune system. This hypothesis is supported by suggestions that surface HSPs may be receptors for NK cells (33-35) and non-conventional T cells, such as Tgamma-delta (36, 37) and $CD4^-CD8^-$ double negative T cells (38, 39), as well as data showing gp96 on the surface of a number of cell types (20, 40, 41)

Hypothesis two: Gp96 can directly transfer peptides to MHC class I on the cell surface. There are two distinct scenarios. First, for antigen positive target cells such as tumor cells or virally infected cells, transfer of surface gp96-chaperoned peptides to MHC I can facilitate direct priming of antigen-specific T cells or potentiate the recognition of target cells by effector T lymphocytes. To date, this possibility still exists and has not been experimentally challenged. Second, transfer of peptides chaperoned by cell surface gp96 on target cells to MHC I of professional antigen presenting cells might result in more efficient cross priming of antigen-specific T cells by APCs. However, this possibility is unlikely to be true, due to the later finding that only receptor-mediated endocytosis of gp96-peptide complex leads to cross-presentation of peptides to MHC class I (42).

Hypothesis three: Peptides bound to gp96 in the ER eventually are released and associated with MHC class I in the target cells. The association of gp96-bound peptides with class I molecules is, in this case, proposed to be due to exogenous or tumor-derived gp96 mimicking the normal cellular role of gp96 within the APC after receptor-mediated endocytosis. Although there is no direct evidence for an involvement of gp96 in antigen presentation to class I molecules, the molecular properties of gp96, including peptide and ATP binding (22, 43-45), subcellular location (19, 46), aminopeptidase activity (47) and association with MHC class I (Z Li and PK Srivastava, unpublished observation), all suggest that gp96 may have a role in conventional antigen presentation which is being exploited during immunization.

Hypothesis four: The presence of gp96-peptide complexes in the ER plays insignificant roles in antigen presentation in a steady-state situation (32). Even in stress and death conditions, such as viral infections and transformation, the presence of enough chaperones in the ER and the requirement for as few as 100 MHC-peptide complexes to activate T cells would suggest that the role of gp96-peptide complexes is not to sensitize target cells for T cell recognition. Instead, the major function of gp96-peptide complexes is to be released or to be accessible to the immune system during stress and death, followed by specific interaction with APCs (30, 32). Such an interaction leads to the activation of APCs and cross-presentation of gp96-associated peptides from target cells to the MHC I of APCs. This highly efficient and regulated process has been shown to be dependent on gp96 receptors such as CD91 (48, 49), CD36

(50) and Toll-like receptors (TLRs) (H Schild, personal communications). This hypothesis has recently received a considerable amount of support. We shall now summarize and examine the supporting data in the following sections.

3. SEARCHING FOR BIOCHEMICAL CLUES FOR THE ROLES OF GP96 IN ADAPTIVE IMMUNITY

3.1. Mechanistic and structural aspects of gp96-peptide interaction

Molecular chaperones are known to associate with polypeptide backbones to facilitate protein folding and unfolding. One such chaperon, HSP70, was studied extensively for its ability to associate with peptides. Using a peptide affinity column, Rothman and colleagues demonstrated direct association of peptides with GRP78, an ER lumenal HSP70 (51). This peptide binding ability was further characterized using a set of random peptides synthesized *in vitro* and testing the abilities of these substrates to stimulate the ATPase activity of GRP78. It was found that the peptide-binding site of GRP78 selects for aliphatic residues and accommodates them in an environment energetically equivalent to the interior of a folded protein (52). Using affinity panning of bacteriophage libraries that displayed random octapeptide or dodecapeptide sequences at the N-terminus of the adsorption protein (pIII), the peptide binding property of GRP78 was further investigated by Gething, Sambrook and their colleagues (53) It was found that GRP78 preferentially binds peptides containing a subset of aromatic and hydrophobic amino acids in alternating positions, suggesting that peptides bind in an extended conformation, with the side chains of alternating residues pointing into a cleft on the GRP78 molecule. This conclusion was supported by the finding that synthetic peptides with sequences corresponding to those displayed by GRP78-binding bacteriophage, bind to GRP78 and stimulate its ATPase activity. The peptide-binding fragment of a highly homologous prokaryotic HSP70 DnaK was structurally resolved (54).

The peptide binding property of gp96 was suggested primarily by the finding that structurally unaltered gp96, purified from tumor cells but not from normal tissues, could immunize for T cell immunity. This suggestion led immediately to the prediction that gp96 should bind to viral antigens, or other model antigens in cells. If so, immunization with gp96 purified from virally infected cells should be expected to induce T cell immunity against viral products. This prediction turns out to be true. Immunization with gp96 isolated from cells infected with SV-40 virus or influenza virus A induces CTLs against cells infected with those viruses (55). It was found that gp96 preparations from vesicular stomatitis virus (VSV)-infected cells elicited a specific CTL response against VSV viral proteins (56). Furthermore, the antigenic peptide (a H-2-K^b restricted 8-mer from VSV nucleoprotein) associated with gp96 was chromatographically demonstrated. Similarly, gp96 isolated from cells expressing beta-galactosidase (beta-gal) and minor H antigens induced CTLs specific for beta-gal as well as minor antigens (57). Up to the present, gp96 has been found to associate naturally with peptides derived from tumor antigens (44), viral antigens (56, 58), bacterial antigens (59), minor histocompatibility antigens (57), and various other model antigens (60) (table 1). Using a microsome based system and cross-linkable peptide substrates, it was found that gp96 is one of the major peptide acceptors in the lumen of the ER, in addition to protein disulfide isomerase (PDI) and GRP170 (61, 62).

In an attempt to define the parameters related to gp96-peptide interaction, it was found, accidentally, that the gp96-peptide complex is thermally stable (63). Incubation of gp96 with a

radioactive peptide at 4°C results in no association of radioactive peptides with gp96. Raising the temperature up to 60°C led to complex formation, as measured by acquisition of radioactive material by gp96. Surprisingly, the complex is stable and not sensitive to SDS, since the radioactive complex can be resolved on SDS-PAGE and visualized by autoradiography (figure 2). This simple heat-dependent folding assay has allowed us to complex exogenous peptides to gp96 with ease. Gp96-peptide complexes reconstituted *in vitro* elicited antigen specific CD8+ cytotoxic T lymphocytes, in a manner that is indistinguishable from native gp96-peptide complexes (63). Using this assay, we have performed cold peptide competition experiments to calculate the association/dissociation constant (Ki) of gp96 in interaction with a 15-mer peptide (KRQIYTDLEMNRLGK) to be 856 pmol (figure 2). By shortening this peptide from either N or C-terminus, one residue at a time to the minimal 5-mer peptides, we generated 20 non-overlapping peptides. Each of these peptides was found to inhibit the binding of gp96 to the 15-mer peptides, albeit at different efficiency (table 2). This data has to be interpreted with caution since the sequence, solubility of the peptides, in addition to the peptide length, might influence the binding to gp96. It does seem that gp96 binds preferentially to 11-mer peptides.

Wearsch *et al.* have initiated a series of studies on the structure/function relationships of gp96 in binding to its ligand. By analyzing a structure of native gp96, they showed that gp96 is an obligatory dimer with an extended, rod-like structure (64). Furthermore, they have identified that a discrete domain, corresponding to amino acid residues 676-719 regulates dimeric assembly and displays autonomous dimerization activity. The saturable, specific and temperature-sensitive peptide binding activity of gp96 was also demonstrated (65). In later studies, Wearsch *et al.* used environment-sensitive fluorescent probes to identify that the hydrophobic pocket in gp96 is a site of peptide binding (65). These results have been incorporated into a structural model of gp96 and provide a framework for future studies on its chaperone activity (figure 3).

Recently, Sastry and colleagues (43) as well as Wearsch *et al* (65) suggested that peptides bind to an open conformation of gp96 in a hydrophobic pocket and there may be a conformational change in gp96 for the loading of peptide. The peptide was found to bind to a hydrophobic region of gp96 in low salt conditions, and to a more hydrophilic region of the protein in high-salt conditions. In a related study, the peptide-binding site was mapped to amino acid residues 624-630 in a highly conserved region and the peptide-binding pocket abuts the dimerization domain of gp96 (figure 3). Pursuing a similar study, Linderoth *et al.* constructed a mutant form of gp96 protein lacking the dimerization domain, and then analyzed the structure of a wild type and a mutant gp96 and their peptide complexes. This result has shown that the bound antigenic peptide was located in a hydrophobic pocket and depletion of the dimerization domain affected the peptide-binding microenvironment (66). By using scanning transmission electron microscopy, it was found that gp96-peptide-complexes exist in higher order multimeric complexes, and some specific aromatic amino acid residues in the gp96 peptide-binding.

Many questions remain regarding the structural basis of gp96-peptide interaction. Studies into the regulation of its interaction both *in vitro* and *in vivo* would be insightful. Extensive mutagenesis, coupled with functional studies in a gp96 null cell line, as well as crystallographic studies, would provide a final answer to these questions.

3.2. ATP binding and ATPase activity of gp96: where is the functional link?

One of the functional hallmarks of many HSPs is their ability to associate with and hydrolyze ATP. In some cases ATP seems to regulate substrate binding, and in other cases it seems to act as a catalyst for rapid protein folding. The ATP binding and ATPase activity of the HSP90 family were controversial until two pivotal papers in 1997 (67,68), which demonstrated, by crystallography, the association of the N-terminal highly conserved 25 kD domain of HSP90 with ATP, as well as the association of the same domain with geldanamycin (GA). GA is one of the ansamycin drugs, which specifically target HSP90 (69) and it inhibits the HSP90 ATPase with nanomolar affinity. The importance of ATPase activity of HSP90 was unequivocally demonstrated by mutagenesis studies in yeast. Despite a slow ATPase activity *in vitro*, a single point mutation to abolish HSP90 activity is unable to rescue the lethal phenotype in yeast (70).

Gp96 is structurally similar to HSP90 (figure 3). Several studies have addressed whether gp96 is also an ATP binding protein and ATPase. Using photoaffinity labeling of purified gp96 to measure ATP binding by gp96, Li and Srivastava found that gp96 interacts with ATP directly *in vivo* (22). Highly purified gp96 was then measured *in vitro* for ATPase activity. Indeed, a Mg^{2+}-dependent ATPase activity was consistently observed. Moreover, this activity was stimulated by misfolded proteins, such as casein, *in vitro*, but not by peptides, suggesting that the ATPase activity of gp96 is closely related to the chaperone function of gp96. Csermely *et al.* also have shown that gp96 has two ATP-binding sites and possesses an intrinsic autophosphorylation activity (71). Nichitta and colleagues contributed extensively to this question using an *in vitro* assay exclusively. They reported earlier that gp96 was able to bind to ATPs, but the low ATPase activity may not be the intrinsic property of gp96 (72). More recently it was shown that an adenosine derivative N-ethylcarboxamidoadenosine (NECA) binds to gp96 with a stoichiometry of 1 mol of NECA per 1 mol of gp96 dimer (73). In addition, NECA binding to gp96 can be wholly competed by geldanamycin, radicical, and ATP, suggesting that NECA binds to the N-terminal adenosine nucleotide-binding domain. These data are consistent with the notion that the N-terminal ATP-binding domain of gp96 is functional equivalent to HSP90, and that gp96 binds to ATP.

The controversy surrounding the ATPase activity of gp96 may stem from the fact that the activity *in vitro* is weak, and it is therefore sensitive to varying conditions associated with assays used by different investigators (16). The ultimate solution would be to precisely mutate the potential residues involved in ATP binding and hydrolysis and study if mutant molecules are associated with functional loss of gp96. Precisely this was done by Randow and Seed recently (74). By subjecting a pre-B cell line to random frame shift mutation and selecting a variant for loss of endotoxin responsiveness, a cell line with both gp96 alleles mutated was serendipitously discovered. This gp96 null mutant is unable to export TLR2 and TLR4 to the cell surface. The phenotype can be rescued by transfecting wild type gp96 cDNA back to the cell, but not by transfecting gp96 mutants with point mutations to disrupt ATP binding and ATPase activity of gp96. It was further shown that gp96 interacts with TLR directly, thus establishing that gp96 is a chaperone for TLRs, and the interaction with ATP is vital for this process.

The other functional link between ATP and gp96 comes from the study of peptide transport into an *in vitro* microsome system (75). By using DIDS (4,4'-diisothiocyanatostilbene 2,2'-disulfonic acid), an inhibitor of the ER-resident ATP transporter and a photo-crosslinkable peptide, it was found that the transport of peptides both into and out of the lumen of microsomes (75) and binding to gp96 and PDI are strictly dependent on ATP (Pieter Spee, personal communication).

3.3. Requirement for further trimming of antigenic peptides in the ER and the potential role for gp96 in the process

MHC class I molecules present peptide antigens to $CD8^+$ T lymphocytes. The assembly of MHC class I - peptide complexes in the ER is a dynamic and highly coordinated process. There is now strong evidence for the idea that a substantial proportion of peptides generated in the cytosol due to proteosomal action are extended on the amino terminus with respect to the MHC I-binding epitopes (76). These peptides need to be further trimmed in the ER, although redundant cytosolic trimming mechanisms have also been demonstrated (77). A number of studies suggest that MHC I guides the trimming in the ER, perhaps by protecting the octamer and allowing the 'overhang' to be acted upon by an aminopeptidase (78), while other studies suggest a lack of MHC I involvement in the process (79, 80). Menoret *et al.* have shown recently that gp96 is an aminopeptidase *in vitro* (47). Gp96 can trim amino terminus extended 19mer precursors of an octamer epitope derived from the vescicular stomatitis virus to the octamer.

The peptidase activity of gp96 needs to be further characterized with regards to the Km and maximum velocity. Furthermore, the regulation of this activity by other molecules such as ATP, other ER chaperones, or MHC itself needs to be considered. The most relevant question to understand is whether the activity observed *in vitro* is operative *in vivo* (see section 5.1). We are actively studying this process by using gp96 null cell lines.

4. IMMUNOREGULATORY ACTIVITIES OF EXTRACELLULAR GP96: UNVEILING THE "DIRTY LITTLE SECRET" OF IMMUNOLOGY?

The challenge for adaptive immunity is to tolerate self, but at the same time to reject non-self, such as virus-infected cells, or altered self, such as malignancy. This specificity is achieved to a large extent by central and peripheral tolerance mechanism by which autoreactive T cells are negatively selected in the thymus (81), and rendered anergic/apoptotic in the periphery (82). Since neither of these two selection processes is perfect, and autoreactive T cells are readily detectable in the periphery, other mechanisms must exist to ensure the appropriateness of immune responses. Recent developments indicate that the context in which antigens are delivered to the immune system plays important roles in the initiation of immune responses. It is proposed that productive immune responses occur only if there is a "dangerous" environment, which harbors natural or endogenous adjuvants (the need for an adjuvant was called "the dirty little secret of immunology" by Charles Janeway, Jr (83) that are normally not present in the microenvironment of healthy tissues (84). Since dendritic cells play central roles in the initiation of adaptive immunity, such endogenous adjuvants are expected to activate immunogenic DCs (85).

4.1. Extracellular gp96 interacts with APCs

The necessity of APCs for gp96 to induce strong T cell immunity came from studies showing that depletion of the phagocytic function of APCs during the immunization phase abolished the effect of gp96 (86). It was soon discovered that macrophage-like cells in peritoneal exudates were able to re-present or cross-present peptides chaperoned by gp96-peptide complexes to their own MHC class I molecules (87). The unexpected potent efficiency of gp96-peptide complex vaccination and the dependence on APCs led Srivastava *et al.* to suggest the presence of receptor(s) for gp96 on APCs (28). By immunofluorescence and electron microscopy, multiple groups have indeed provided convincing evidence for receptor-

dependent interaction of gp96 with macrophages, dendritic cells and B cells (88-91). Furthermore, by using affinity purification with gp96-conjugated column, Binder *et al.* purified the cell surface ligand (receptor) for gp96 from a macrophage cell line, RAW 264.7 to homogeneity. A polyclonal antibody raised against this protein can block the re-presentation of gp96 chaperoned peptide to MHC class I (91). Microsequencing of this molecule by mass spectrometry confirmed it to be CD91, a protein known as alpha 2-macroglobulin receptor or the low-density lipoprotein-related protein. CD91 as a receptor for gp96 was further supported by the evidence that alpha 2-macroglobulin, a previously known CD91 ligand, inhibited re-presentation of gp96-chaperoned antigenic peptides by macrophages, as did antibodies against CD91 (49, 91). It is possible that CD91 is not the only receptor that gp96 can bind, given the fact that the interaction of gp96 with APC triggers a cascade of events that are important for productive immunity.

4.2. Peptide-independent activation and maturation of dendritic cells by gp96

Agents that activate and mature DCs can be broadly classified into two groups: exogenous agents typified by bacterial product LPS (92) and unmethylated CpG molecules (93, 94); endogenous agents such as inflammatory cytokines IL-1 beta and TNF-alpha (95). In the last two years, gp96 and other members of HSP family have emerged as the likely universal DC activators.

The presence of universal (expressed in every cell) endogenous DC activators has long been postulated. Such molecules are expected to be present only in a situation when an immune response is needed. Gallucci *et al* showed that necrotic cells, but not healthy or apoptotic cells can mature bone marrow derived murine DCs (96). Later, Sauter *et al.* showed that human DCs could also be activated by necrotic cells or their supernatant (97). These observations are in agreement with the notion that abnormal death (necrosis) is proinflammatory due to the

activation of DCs by soluble molecules liberated. Recent studies suggested that HSPs are the molecular definition of these proinflammatory molecules. Basu *et al* showed that necrotic cells, but not apoptotic cells, release HSPs including gp96, HSP70, HSP90 and calreticulin (98). They also found that soluble gp96 purified from normal tissues, can stimulate bone barrow derived DCs to express MHC-II and the co-stimulatory molecule B7.2, whereas non-HSPs were not able to do so at the same concentration (49, 89, 98). The activation of DCs by soluble HSPs has been independently confirmed by many groups in human and murine system, supporting the hypothesis that HSPs are the sensors for non-physiological death because they are the endogenous activators of DCs (table 3).

Cellular trafficking of gp96 has also been shown to have an impact on immunity. In non-stressful conditions, gp96 resides in the lumen of the ER. Upon stress, both surface expression and secretion of gp96 have been reported (20, 46) Although the cell biological basis of gp96 "ectopic" transport is unknown, its effect on an immune response has been studied. By deleting carboxyl terminal KDEL ER retention signal and fusing with the Fc fragment of immunologlobulin, Podack and his colleagues have engineered a gp96 secretory molecule. Tumor cells secreting gp96-Ig were found to be much more immunogenic (99). Similarly, by fusing a transmembrane domain with the C-terminus of gp96, Zheng *et al* have found that gp96 surface expressing tumor cells induced robust maturation and activation of DCs, as evidenced by up-regulation of MHC-II, CD80, CD86, CD40, and secretion of pro-inflammatory cytokines such as IL-1 alpha, IL-12 and chemokine MCP-1 (100). The activation

of DCs by HSPs such as gp96 mirrors the effect by lipopolysaccharide (LPS). It was shown that gp96 activates DCs at least partially through NF-kappa B, although the kinetics is clearly different from that of LPS (98).

4.3. Gp96 induces the migration of dendritic cells to lymphoid organs

Migration of DCs to secondary lymphoid organs is an essential step in priming an adaptive immune response. Accumulating evidence demonstrates that the capacity of DCs to migrate to draining lymph nodes is mediated by chemokines interacting with chemokine receptors (101-103). Inflammatory chemokines, such as MIP-3 alpha (104), MIP-1 alpha and MCP-1 (105) are responsible for recruiting cells to inflammatory sites, while constitutive chemokines, such as MIP-3 beta (105), are responsible for the trafficking of immune cells to lymphoid organs. Immature DCs express high levels of receptors for inflammatory chemokines, such as CCR1, CCR2, CCR5 and CCR6, which guide them to the inflammatory site, where they pick up antigens. Upon maturation, receptors for constitutive chemokines, such as CCR7, are up-regulated, which leads to the migration of DCs to secondary lymphoid organs, where DCs prime naïve T and B cells (104, 106).

Soluble gp96 can induce migration of DCs to draining lymph nodes *in vivo* (107). Immunization of mice with as little as 1 mug of gp96 induced a 5-7 fold enlargement of the draining lymph nodes within hours after injection. This enlargement is the result of accumulation of CD11c+ cells. These presumed Langerhan's cells obtained a mature phenotype and were able to stimulate naïve T cells. DCs activated by cell surface-bound gp96 also have enhanced migratory ability to chemokine MIP-3beta (H Zheng and Z Li, unpublished observation), indicating that chemokine-chemokine receptor interaction is involved in gp96 induced migration of APCs.

4.4. Chaperoning antigenic peptides by gp96 to MHC I of antigen presenting cells: fulfilling a role in cross-presentation?

In general, endogenous antigens are presented to MHC I by non-professional APCs for recognition by CD8+ T cells, whereas exogenous antigens can be picked up, processed and presented to the MHC class II pathway by APCs (108). Since non-APCs do not prime $CD8^+$ T cells due to lack of expression of co-stimulatory molecules, antigens from these cells have to be cross-presented to the MHC I of APCs (109). This cross-presentation pathway is necessary for priming of $CD8^+$ T cells against tumor antigens (110), viral antigens (109) and a variety of model antigens (111). When soluble HSPs, including gp96, were found to immunize effectively for tumor-specific CTLs without the use of an exogenous adjuvant, it was immediately suggested that gp96 plays an important role in cross-presentation (28). Indeed, it was found that exogenous peptides chaperoned by gp96 were presented to MHC I by Pristane-induced peritoneal macrophages (87). Furthermore, gp96 isolated from VSV infected cells of $H\text{-}2^d$ background could induce $H\text{-}2^b$ restricted VSV-specific CTLs, demonstrating that antigens chaperoned by gp96 can be cross-presented to MHC I of APCs (87). Similar findings were obtained using other model antigens such as minor histocompatibility antigens and beta-galacatosidase (112). Impressively, compared with soluble peptides, the amount of gp96-peptide complexes required to prime T cell response is several orders of magnitude less (63). As discussed previously, this unusual high efficiency led to the suggestion and eventual confirmation that cross-presentation of gp96 chaperoned peptide is receptor mediated.

4.5. Characterization of the gp96 receptors

As described in Section 4.1, interaction of gp96 with its receptor(s) on APCs is expected to induce profound molecular events, leading to the maturation, activation, migration of DCs, as well as cross-presentation of gp96-chaperoned antigens. Not surprisingly, energy was focused on cross-presentation and the identification of key molecules in this process. Using affinity chromatography, the receptor responsible for cross-presentation of gp96 was identified as CD91 (48). Antibodies to CD91 completely inhibited the re-presentation of peptides bound to gp96, supporting the previous evidence that, without receptor-mediated endocytosis, gp96-peptide complexes cannot initiate cross-presentation (42). Further studies by Basu, *et al.* showed that CD91 is a receptor not only for gp96, but also for other peptide chaperones known to induce immune responses via cross-presentation of antigens, namely, calreticulin, HSP70 and HSP90 (49).

It is doubtful that CD91 is also a functional receptor for mediating the effects of gp96 in maturing and activating DCs, since CD91 itself has limited signaling capacity. Interestingly, using a mutant cell line that is defective of signaling by LPS, it was found that gp96 binds and chaperones TLR-2 and TLR-4 selectively, presumably in the lumen of ER (74). This finding raises the possibility that extracellular gp96 interacts with cell surface TLRs for signaling. Indeed, by using various TLR knockout mice and *in vitro* transfection of TLR genes into HEK293 cells, it was shown that gp96 utilizes both TLR2 and TLR4 for signaling (H Schild, personal communication). This data could correlate nicely with the observation that gp96 stimulation of DCs and macrophages leads to translocation of NF kappa B to the nucleus (98). This transcription factor could be extremely important in the downstream effects of gp96 stimulation described above. The toll-like receptors are attractive candidate receptors for gp96 because, like gp96, their function is primitive, and their evolutionary conservation is great. The importance of toll-like receptors in innate immunity supports the hypothesis that gp96 was an important molecule in host defense long before the adaptive immune system developed.

In addition to TLRs and CD91, there is evidence that the scavenger receptor CD36, present on macrophages and other phagocytic cells, can also interact with gp96. One of the clues is that the gp96 receptor(s) as well as CD36 were down-regulated on the surface of mature dendritic cells (89). Evidence for CD36 as a receptor for gp96 includes: CD36 null macrophages bind 52% less gp96 than their $CD36^+$ counterparts, and cells transfected to express human CD36 bound more gp96 than untransfected, $CD36^-$ cells (50) (Srivastava PK, personal communications). CD36 also has the potential to be a signaling receptor based on the presence of one tyrosine kinase domain sequence within its cytoplasmic tail (50).

The details of gp96 effects due to receptor-mediated endocytosis and receptor signaling will most definitely become clearer very soon. Such knowledge undoubtedly will help us understand the basis and regulation of the complex interactions of gp96 with APCs. It may also open doors for generating novel pharmacological reagents for manipulating immune responses for therapeutic purpose.

4.6. Immunological circuit as a result of gp96 vaccinations

An insight into the mechanisms through which gp96 interacts with the immune system to induce antigen-specific cellular immunity was first made by Udono *et al* (86). Mice immunized with irradiated intact Meth A cells required $CD4^+$ T cells during the priming phase to generate

an anti-tumor response; in contrast, mice immunized with soluble gp96 derived from Meth A cells required $CD8^+$ T cells but not $CD4^+$ T cells for priming (table 4). However, in the effector phase of tumor rejection, both $CD4^+$ and $CD8^+$ T cells were necessary. In both the priming and effector phases, phagocytic cells were necessary for gp96, but not for whole cell vaccinations. This is consistent with the requirement of APCs to cross-present gp96-bound peptides.

Several independent groups further documented that gp96-peptide complexes are capable of inducing antigen-specific $CD8^+$ and $CD4^+$ T cells. In these *in vivo* and *in vitro* experiments, a broad range of models has been employed, covering mouse and human tumor models, as well as native and reconstituted gp96-peptide complexes (table 1). Using an MHC-II restricted $CD4^+$ T cell clone against a mutated peptide from the ribosomal protein L11 (113), it was found that gp96 can also associate with this peptide or its precursor(s) (Srivastava PK, personal communications). Pulsing of APCs with gp96-peptide complexes isolated from Meth A containing the mutant protein effectively stimulated the proliferation and cytokine production of IL-5 by an L11-specific clone. The effect could be inhibited by anti-CD91 antibody, suggesting that the presentation of MHC II epitope associated with gp96 utilizes the same upstream molecules.

Tumor cells engineered to secrete gp96 are more immunogeneic in multiple tumor models (99). It was demonstrated that anti-tumor immunity induced by tumors secreting a gp96-Ig fusion protein was dependent on $CD8^+$ T cells without the need for $CD4^+$ T lymphocytes.

Similarly, by attaching a transmembrane domain to gp96 and transfection, Zheng *et al.* have been able to target gp96 expression to cell surface. A single immunization with gp96 surface-expressing tumor cells stimulates IFN-gamma-producing lymphocytes with high-frequency, leading to tumor regression (100). Interestingly, this immunity is dependent on both $CD4^+$ and $CD8^+$ T cells. Phagocytic cells, however, are not required (J Dai and Z Li, unpublished observation). These studies suggest that surface gp96 might prime tumor-specific immunity in a distinct mechanism, perhaps as a result of high avidity interaction of surface gp96 with gp96 receptors due to cross-linking, or perhaps due to direct exchange of peptides chaperoned by gp96 to MHC I and MHC II. Alternatively, the differential requirement for phagocytes, in addition to $CD4^+$ or/and $CD8^+$ T cells, might be related to different tumor models or/and different immunization protocols (table 4). Recent studies suggest that cross-presentation of cell-based antigens is more efficient than that of soluble antigens (114). The doses of gp96 and routes of immunization also have a profound impact on HSP induced immunity (section 5.3) (115). Higher than optimal doses of gp96 can even down regulate anti-tumor immunity. Therefore, careful comparison among different vaccination strategies of gp96 is necessary to understand the underlying mechanisms, as well as for future clinical applications.

Whether or not gp96 can interact with B cells remains unclear. The existence of a phagocyte-independent antigen presentation pathway, the gp96 receptor on B cells (42), together with the findings that peptides associated with gp96 can be re-presented via both MHC-I (87) and MHC-II pathways (T Matsutake and PK Srivastava, personal communications) leads to speculation that B cells could be directly or indirectly involved in this process. Binding of gp96 to B cells has been observed (42) by using fluochrome-labeled gp96. Navaratnam *et al.* reported that by immunizing with gp96 isolated from cells expressing glycoprotein D of bovine herpesvirus 1(BC-gD), mice were able to generate specific CTLs and antibodies against BC-

gD as well (116). Interestingly, natural autoantibodies (IgD) for gp96 exist at a low but sustained level, which couldn't be further boosted by immunization with gp96 from the livers of syngeneic mouse (117). Meanwhile, antibodies to HSP90, a cytosolic paralog of gp96, play dual roles in down regulating antigen-specific T cell responses and activation of peripheral blood mononuclear cells. These observations highly suggested that autoantibodies are involved in maintaining balance between activation and inactivation of HSP-induced immune responses and that the production of antibodies is finely regulated by as yet unknown mechanisms.

It has been reported recently that platelets also expresses high level of CD91, a common HSP receptor (H Schild, personal communication). Platelets are small, non-nucleated cell fragments derived from myeloid progenitors that are important for hemostasis. The association of the actin-rich cytoskeleton with rapidly phosphorylated HSP27 has been shown when platelets were activated (118). A phosphorylated complex of HSC70, HSP90, and the catalytic and myosin-targeting subunits of protein phosphatase 1 has been demonstrated to undergo rapid disassociation and dephosphorylation after adhesion of platelets to collagen (119). These results indicate that HSPs may be involved in actin polymerization or serving as signaling scaffolds. It was found that gp96 is able to bind to human platelets specifically through CD91. Binding of gp96 to platelets does not interfere with the intrinsic function of platelets, such as activation and aggregation induced by ADP or collagen (H Schild, personal communications). However, the presence of platelets inhibits gp96-induced DC maturation *in vitro*, suggesting that the sequestration of gp96 by platelets *in vivo* may provide a negative regulation of immune responses. Such an anti-inflammatory response might be helpful for wound healing, therefore the physiological function of the interaction between gp96 and platelets needs further investigation.

5. AN INTEGRATED VIEW OF GP96 IN IMMUNE RESPONSE

5.1. Exploring the role of gp96 in antigen presentation

It has long been known that MHC class I molecules contain peptides, however, the details of their source and the path that they follow before binding the MHC in the endoplasmic reticulum has proven to be quite complicated. We now know that most peptides degraded in the cytosol come from newly synthesized proteins (120-122). The proteosome and immunoproteosome components that participate in this degradation pathway have also been well characterized, although their specificity in generating class I peptides remain somewhat mysterious. After proteosome processing, these defective ribosomal products (DRiPs) become the source of peptides that get loaded onto MHC class I molecules in the ER. Despite the rapid and continuous production of these peptide products in the cytosol, very few peptides can be isolated from the cytosol of a normal cell. To explain this, it has been postulated that abundant chaperones in the cytosol, including HSP90, participate as a relay and sink for newly generated peptides (28). It is known that the TAP heterodimer on the ER membrane is responsible for shuttling peptides from the cytosol into the ER lumen. To date, there is no reported association of HSP90 with TAP. The evidence for the association of HSP70 with TAP is weak, so the exact details of which peptides get delivered from the cytosol and how are still unclear (123). As discussed previously, there is also evidence for resident cytosolic and/or ER aminopeptidases, given that the proteasome creates peptides with carboxy termini exhibiting high affinity for the MHC class I peptide binding site, but amino termini that are not ideal for recognition by class I. Indeed, two new cytosolic, proteosome-independent aminopeptidases were recently identified (77).

A role for gp96 in antigen presentation was proposed as early as in 1991. Supporting evidence continues to emerge, although no definitive evidence has materialized. There are several factors which implicate gp96 as a candidate in the presentation of peptide antigens onto class I molecules (29, 32). First, gp96 is extremely abundant in the endoplasmic reticulum. It makes up approximately 3% of all ER proteins, making the likelihood of interaction with class I molecules very high. In fact, in cells where there is a disproportionately abundant amount of empty MHC class I, the association of MHC I with gp96 can be demonstrated (Z Li, unpublished observation). Second, gp96 is known to avidly bind peptides (section 3.1), suggesting that gp96 could be a carrier for peptides being delivered by TAP, holding onto them until the class I molecules have reached the correct conformation to allow their binding. Third, although the peptide-binding specificity of gp96 is not nearly as selective as that of class I, gp96 has been implicated as an aminopeptidase *in vitro* (47). As stated above, the proteosome does not always generate peptides of the correct length and sequence to bind class I molecules, especially at the amino terminus. Therefore, there is a requirement for an aminopeptidase either in the ER or the cytoplasm. Although the aminopeptidase activity of gp96 *in vitro* was relatively low, it is reasonable to assume the kinetics would be enhanced in the lumen of the ER, given that the ER lumen has carefully regulated osmotic conditions, pH, and accessory molecules that an *in vitro* system cannot replicate. Therefore, gp96 could be cleaving either MHC I-bound peptides, or peptides it binds before handing them over to class I molecules. Fourth, gp96, like other molecules in the class I antigen presentation pathway, is up-regulated in response to IFN-gamma stimulation (124). This fact indicates that gp96, along with MHC I heavy chain, TAP and immunoproteasome components, is coordinately regulated during viral infection. Fifth, gp96 harbors ATPase activity (22), which has recently been implicated in its functional abilities (74), and which supports an active role within the ER. Given that the assembly of MHC I-peptide complex is ATP-dependent (125), ATP binding and ATPase activity of gp96 therefore also fulfills a functional requirement in assisting antigen presentation. Sixth, gp96 has the ability to cross-prime, as described above, indicating that it can somehow deliver peptides to class I MHC molecules when delivered exogenously. Although it is still unclear whether this exchange of peptides from exogenous gp96 to MHC class I molecules happens directly, either in an endosomal compartment or in the ER via retrograde transport of endocytosed gp96-peptide complexes, or indirectly, via release of gp96-bound peptides into the cytosol to be transported into the ER, the mere fact that endocytosed gp96-bound peptides can be represented on class I molecules lends strong support for a role for gp96 in presentation of normal endogenous antigens.

There are, however, some data that argue against a role for gp96 in antigen presentation. First, cells expressing high levels of antisense cDNA of gp96 constitutively expressed drastically reduced level of gp96 protein. These cells failed to exhibit decreased MHC class I surface expression or inability to be lysed by antigen-specific T cells (126). This experiment was limited by the inability to convincingly eliminate 100% of the gp96 protein and by the fact that only one antigenic epitope was tested, that for VSV nucleocaspid protein. Second, a recently identified mutant pre-B cell line that has two truncated gp96 alleles failed to exhibit a noticeable deficit in class I surface expression, however, the structural conformation and peptide binding ability of the detected class I molecules was not described (74). While there is also no evidence that gp96 associates with any of the molecules that have been identified so far in the MHC complex (TAP, Tapasin, calnexin, calreticulin, or Erp57), the often used immunoprecipitation in the presence of detergent might not be the optimal assay for studying protein-protein interaction. It could also be argued that the abundance and phylogenetic conservation of gp96, all the way back to cells and organisms that have no adaptive immunity

or antigen presentation systems, indicate its main role is probably more fundamental than that of an ER protein required for antigen presentation. However, it is not beyond the realm of possibility that as adaptive immunity and antigen presentation evolved, such systems made use of this protein, given that it is so abundant, and is a peptide-binding protein.

Our discussion so far is limited to the role of gp96 in *de novo* presentation of endogenous antigens to MHC I pathway. Cross-presentation of gp96-chaperoned peptides to MHC I of APC requires receptor-mediated endocytosis, and translocation of either gp96 or gp96 ligand across the plasma membrane to cytosol, since it has been shown that cross-presentation is dependent on TAP and the proteasome (49). The cell biology basis is unclear. An experiment to allow visualization of the peptides bound to gp96 as they enter the cell would be the ideal way to discern the details of the endocytic pathway, however, this is difficult to do with current technology. Electron microscopy has shown that endocytosed gp96 localizes to non-acidic endocytic compartments and colocalizes with class I and class II molecules (88). Whether this colocalization is enough to allow peptide exchange is unknown. Retrograde transport is another possible mechanism by which gp96 could be mediating cross-priming. There are examples of molecules that find their way to the trans-golgi network via endocytosis mediated by special receptors and then are taken back to the ER by retrieval proteins which recognize their KDEL retention sequences. The *E. coli* toxin VT-1 and the Shiga toxin, ricin, are two such proteins. Given that gp96 has a KDEL signal, retrograde transport of endocytosed gp96-peptide complexes by KDEL receptor is an attractive mechanism for cross-presentation.

5.2. Gp96 (and other HSPs) are the molecules in cross-priming the adaptive immunity

Intracellular antigens in non-APCs, such as tumor antigens or viral antigens, have to be cross presented from these cells to the class I molecules of APCs for the priming of $CD8^+$ T cells. The requirement for cross-priming by APCs is dictated by two considerations: First, only APCs have the capacity to express essential co-stimulatory molecules for T cell activation; second, naïve T cells generally stay in secondary lymphoid organs, whereas APCs are localized in all tissues and organs for sampling antigens. Priming of CTLs across MHC I was operationally and functionally defined by Bevan using minor histocompatibility antigens (127, 128). This phenomenon has now been substantiated in T cell responses against tumor antigens, viral antigens, bacterial antigens and other model antigens (section 4.1.3). The molecular basis for it has now been studied. All data supports the notion that only bone marrow-derived APCs are able to cross-present exogenous antigens. The nature of antigens also dictates whether an antigen can be cross-presented or not. In general, cross-presentation of soluble antigens is extremely insufficient, unless there is a specific receptor expressed on the surface of APCs. For antigens captured by antibody, the Fc receptor on APCs might serve this purpose (129). For a vast majority of intracellular antigens, a generic system including carrier molecules for antigens and the shuttling of antigens through a common receptor for the carrier might suffice. The candidate carrier molecules responsible for such a mechanism are expected to have the following features: universal expression in all somatic cells, binding non-selectively to intracellular antigens, specific interaction with APCs, and capacity to escort exogenous antigens to MHC class I of APCs. As evident from Section 2, HSPs including gp96 possess all of these features and are thus the best candidate molecules so far for chaperoning peptides from non-APCs to MHC class I of APCs.

As discussed previously, the best evidence for the role of HSPs in cross-presentation is the presence of receptors for HSPs on the surface of APCs. CD91 binds directly to gp96, and is functionally involved in presenting peptides chaperoned by gp96 to MHC I molecules *in vitro*.

In addition, HSPs including gp96 are able to stimulate DC maturation as indexed by increased expression of cell surface activation markers, such as MHC I, MHC-II, CD40, CD80 and CD86 (table 3).

Since HSPs are intracellular proteins, the presence of an HSP receptor on APCs and the proposal that HSPs are involved in cross-presentation must mean that HSP-peptide complexes have to be released extracellularly. There are several possibilities. First, release of HSPs occurs after cell lysis. It has been shown that HSPs are released after necrosis, but not after apoptosis (98). This is consistent with the notion that only non-physiological cell death is proinflammatory. Apoptosis is generally a physiological process that is not expected to trigger immune responses. Second, active extracellular expression such as secretion and cell surface expression should also be expected to prime immune response as suggested by cellular engineering (99, 100). Both surface expression and secretion of gp96 by tumor cells have been shown to increase the immunogenicity of tumors. In stress conditions, it was found that surface expression and secretion of gp96 is an active process, and not the result of cell death. It is unclear which pathway (cell death or active cellular transport) plays predominant roles in cross presentation, the clear knowledge on the molecular basis of extracellular trafficking and the regulation of it would be instrumental to our understanding of the roles of HSPs in immune response *in vivo*.

Are HSPs essential for cross-priming *in vivo*? This question has proven to be difficult to address. The description of a gp96 null cell line may help to address this question, although it is virtually impossible to eliminate all HSPs from a cell line. Cell lines with defective TAP can still prime, indicate that ER HSPs such as gp96 are not essential (130). An alternative approach would be to perform the same tedious biochemical experiment that led to the discovery of HSPs as "tumor rejection antigens". If whole cell lysate can cross-prime, biochemical fractionation and characterization of each fraction for their abilities to cross prime T cells would lead to the discovery of the active components necessary for this process. Generation of transgenic animals that export HSPs might also lead to some clues as to whether the interaction of HSPs with their receptors might have immunological consequences.

5.3. Gp96 (and other HSPs) regulate the immune response

The roles of HSPs in the effector phase of an immune response have not been looked at closely. Moreover, studies so far are heavily biased towards the study of HSPs in productive cellular immunity. At least in one scenario, it was found that immunization with higher than optimal dose of gp96 might lead to immunosuppression (115). Therefore, it would be prudent to examine the possibility whether or not gp96 also performs immunoregulatory functions.

Recently, the suppressive or regulatory functions of $CD4^+CD25^+$ T cells have attracted a considerable amount of attention (131). These cells produce high levels of IL-10, IL-4 and TGF-beta upon stimulation, which suppress proliferation of other T cells in both a cytokine (132) and cell-cell contact-dependant manner (133). Another subset of regulatory cells has been cloned and described by Zhang and colleagues (134). These cells express a unique combination of cell surface markers (alpha beta-TCR^+ $CD4^-$ $CD8^-$ $CD25^+$ $CD28^-$ $CD30^+$ $CD44^-$) and secrete a distinct profile of cytokines (IFN-gamma, TGF-beta, TNF-alpha) from that of Th1, Th2, or $CD4^+$ $CD25^+$ regulatory T cells. Inhibition of $CD8^+$ T cells occurs by the Fas-FasL pathway, but only if the target cells have the same TCR specificity.

Another subset of regulatory cells is NKT cells which express alpha beta TCR as well as NK cell receptor (135). Their development is dependent on MHC-I like CD1 molecules (135). NKT cells are highly heterogenous population, at least three subsets ($CD4^+$, $CD8^+$, $CD4^-CD8^-$) of NKT cells have been identified (136, 137). Functionally, they can regulate immune response both positively and negatively. For example, it has been shown that NKT cells are essential in IL-12 mediated tumor rejection (138, 139), indicating their role in positively regulating immune response. On the other hand, Terabe *et al.* found $CD4^+$ NKT cells could suppress tumor immunosurveillance through the production of IL-13 (140). NKT cells can also prevent autoimmune diseases, such as IDDM, by producing IL-14 or IL-10 (141), supporting that they also play inhibitory roles in regulating immune responses.

There is also evidence suggesting that gamma delta T cells can function as regulatory cells (142). Gamma delta T cells can contribute to the resolution mechanism of pathogen induced inflammatory immune response by killing activated inflammatory macrophages . Also, a population of regulatory gamma delta T cells which have a similar cytokine profile to $CD4^+CD25^+$ regulatory T cells has been identified (143). These cells can be found in tumor infiltrating lymphocytes, which inhibit anti-tumor immunity (144).

Limited data indicate that gp96 may contribute to the regulation of these regulatory cells. Chandawarkar *et al.* have found that immunization with optimal doses of tumor-derived gp96 elicits tumor immunity (115). Immunization with a 5-10 fold higher dose of gp96 actually down regulates anti-tumor immune responses. This regulatory effect is antigen specific and is dependent on $CD4^+$ T cells as demonstrated by adoptive transfer. Further characterization of these cells has not been performed, leaving the question as to whether these T cells are $CD25^+$ regulatory cells or NKT cells. It is also unclear how these T cells are generated, whether gp96 interacts directly with these T cells, and what is the mechanism for the suppressive function. Studies also suggest that NKT cells express some level of CD91 (H.L. Aguila, personal communication), indicating that gp96 might interact with NKT cells.

HSPs such as gp96 can clearly modulate the immunological climate by inducing cytokine production by DCs. Efforts so far have been focused exclusively on the induction of APCs to produce pro-inflammatory cytokines such as IL-1, IFN-gamma, TNF-alpha and IL-12. In order to gain some clues as to how HSPs interact with the immune system, it would be insightful to analyze down stream signaling events, and profile all the potential cytokines or other target molecules induced or suppressed by HSPs on a large scale.

6. THE CHALLENGES AND PERSPECTIVES

The unfolding of the story of gp96 is exciting. The unraveling of the details has proven to be nothing but a challenging task both conceptually and experimentally. An open-minded approach is crucial as we put together all the pieces of the gp96 puzzle.

6.1. Conceptual challenges

The immunological roles of gp96 must be examined dynamically. Examination of the context in which gp96 positions itself in the immune response holds the key for resolving the confusions currently surrounding gp96. It is worthwhile to discuss a few examples of conditions in which gp96 may play complimentary or even distinctive roles in the generation of immune responses.

6.1.1. APCs vs. non-APCs

There are two obvious differences between APCs and non-APCs. APCs are the only cell types that can present exogenous antigens to MHC molecules, a process defined as cross-presentation. The second difference is the ability of APCs to express co-stimulatory molecules that are required to activate naïve T cells. Non-APCs do not cross-present antigens and rarely express co-stimulatory molecules. These functional differences between APCs and non-APCs would require distinct roles of gp96 in antigen presentation and re-presentation. For example, gp96-associated peptides can only be cross-presented by APCs. This apparent cell type selectivity cannot be attributed solely to the expression of gp96 receptor CD91, since CD91 is also expressed on non-APCs such as fibroblast, platelets and hepatocytes. It is possible that only in APCs there exists a specialized compartment where MHC I, MHC II and CD91 are in close enough physical proximity for peptide exchange to occur. It is equally possible that there is a retrograde transport mechanism of gp96-peptide complex from early endosome to the ER that is unique to APCs. Moreover, there could be more efficient direct transfer of gp96-chaperoned peptides to MHC I in professional APCs. Finally, *de novo* antigen presentation to MHC I has not been carefully compared between APCs and non-APCs. The possibility remains that molecular chaperones may play more important roles in antigen presentation in APCs than in non-APCs.

6.1.2. Extracellular vs. intracellular gp96

We have now begun to appreciate the differences between extra cellular gp96 and intracellular gp96 in the presentation of antigens. Intracellular gp96 is likely important for all cells, whereas extra cellular gp96 exerts its function simply through APCs. While the distinction is acknowledged, there seems to be a tendency in the field to make at least two assumptions. First, the generation of extra cellular gp96 is simply by a messy cell death, referred to as necrosis. A well-described phenomenon of secretion and surface expression of gp96, particularly under stress conditions, has not received adequate attention. Consequently, there has not been a careful study on the cell biological basis of "ectopic" transport of gp96 and the regulation of it in the context of immune response. Second, the trafficking of extra cellular gp96 and intracellular gp96 in APCs is entirely distinct. There has not been enough emphasis on possible convergence of these two pathways in APCs. Dissecting these two pathways carefully will be instrumental to our understanding of the roles of gp96 in antigen presentation.

6.1.3. Priming vs. effector phase

Much energy has been devoted towards the understanding of the roles of gp96 in priming, a process defined as activation and expansion of naïve antigen-specific T or B cells. However, little attention has been paid to the role of gp96 in the effector phase of an anti-tumor immune response. For example, the presence of anti-gp96 antibody has been described. In the case when gp96 is expressed on a cell surface, anti-gp96 antibody might contribute to the elimination of these cells. It was reported that a gp96 receptor exists on APCs and B cells, but not T cells. But it is unclear if the receptor expression is regulated during the activation of T cells. Future work needs to examine this question, and to test if gp96 receptor can be expressed by other cell types such as NK cells and gamma delta T cells.

6.1.4. Steady state vs. stressed situation

Induction of HSPs by heat has been shown to facilitate migration of DCs to the draining lymph nodes and potentiate the ability of DCs to prime T cells. Moreover, heat shocked cells have been shown to have increased immunogenicity. Therefore, both the expression level and peptide repertoire associated with HSPs such as gp96 are likely to be different between resting and stressed cells. In the acute stress conditions such as virus infection and rapid proliferation of malignant cells, the demand for efficiency of antigen presentation is high, the roles for accessory molecules might therefore become more significant. During non-stressed steady state conditions, the roles of HSPs in direct antigen presentation might be subtle. There is even a possibility for constitutively expressed HSPs in non-stress conditions to deliver tolerogenic signals to the immune system, and thus contribute to peripheral tolerance. Thus, the roles of gp96 have to be analyzed in the context of stressful, or pathological conditions.

6.1.5. Quantity vs. quality

As eloquently pointed out by Jonathan Yewdell, immunologists and cell biologists have profound tendency to focus on the qualitative, rather than the quantitative aspects of cell physiology (145). In the non-disturbed situation, approximately one-third of newly synthesized proteins (approximately $5x10^6$ proteins per minute) are degraded (termed DRiPs, for 'defective ribosome products') by proteasome within minutes of their synthesis. Since MHC I-peptide complexes are formed in the ER at a rate of roughly 150 per minute, 10,000 proteins, on average, are degraded for each class I-peptide complex generated. This suggests that the production of MHC I-associated peptides is an extremely inefficient process in the steady state, which raises a question as to how then a viral or tumor antigen can be rapidly presented in the stress conditions. It is safe to say that the dynamics of protein synthesis, turnover, and transport in these stress conditions would be very different from the resting state. The tremendous inductions of HSPs could surely contribute to the regulation of these dynamic processes. But to sort out the contributions of gp96 or other HSPs, it is clear that qualitative science is not enough. The kinetics of peptide generation, MHC I folding and transport, as well as the accumulation of all the intermediate products have to be carefully analyzed and quantified, preferentially by comparing cells that have normal and no induction of HSPs.

6.2. Experimental challenges

The difficulties surrounding gp96 also stem from numerous experimental challenges, as described below. These challenges must be overcome by ingenious experimental approaches so that a verdict on the roles of gp96 in immune response can be delivered.

6.2.1. Essential non-immunological functions:

Gp96 associates with a broad spectrum of substrates in the secretory pathway. This apparent "lack" of specificity is probably because of its general roles in facilitating protein folding. This is is supported by the finding that disturbance of protein homeostasis in the ER leads to further induction of gp96 expression. Direct introduction of misfolded proteins to the ER strongly induced the synthesis of gp96. In addition to its broad substrate binding capacity, gp96 has also been suggested to have the following enzymatic activities: endo-beta-D-glucuronidase (heparanase) (146), ATPase (22), aminopeptidase (47), and foldase (to facilitate protein folding). Therefore, unlike tapsin or TAP (transporter associated with antigen presentation), gp96 most definitely has multiple non-immunological functions. It is experimentally difficult to

separate the generalized functions of gp96 in the ER from its more specialized immunological functions.

6.2.2. Functional redundancy

The HSP family is composed of numerous members. In the lumen of the ER where gp96 normally resides, there are multiple HSPs that have similar biochemical properties to gp96, such as GRP78, calreticulin, calnexin and GRP178, protein disulfide isomerase. Although each of the players has been found to perform somewhat unique roles in antigen presentation, the roles of gp96 have not been finalized. Even if a gp96 negative cell line is used, it is not clear whether the loss of gp96 can be functionally compensated by other molecular chaperones. This clearly poses an experimental challenge.

6.2.3. Lack of genetic tools

Gp96 is one of the most abundant molecules in the lumen of the endoplasmic reticulum. Polymorphisms or natural mutants of gp96 have not been found. The ubiquitous and constitutive expression suggests that gp96 possibly plays an essential function in cells. This is probably the reason why the manipulation of the expression of gp96 remains difficult. Anti-sense cDNA constructs targeting to the coding sequence (126) or promoter region of gp96 (147) have met with some limited success in a transient system. Homologous recombination has been used to generate gp96 null mice. Unfortunately, gp96 null embryos die very early at embryonic day of 5.5, which has prohibited further study of gp96 function at the organismal level (Rinni de Crom, personal communications). By subjecting a pre-B cell line to a frameshift mutation *in vitro*, a cell line with both alleles of gp96 truncated at the c-terminus has been described recently (74). Conditional gp96 knockout mice using the cre-lox P system are being generated. This new development will undoubtedly facilitate our research in dissecting both immunological and non-immunological roles of gp96.

7. CONCLUSION

The immunological roles of gp96 were discovered serendipitously. The pieces of the puzzles of gp96 in interacting with peptides and dendritic cells have now been placed, and substantiated. Putting all the pieces together has proven to be challenging, however rewarding the process may be. But it is our belief that the extra cellular presence of heat shock proteins like gp96 plays important roles in alarming, mobilizing and activating the immune system in the face of cancers, infections and other hazardous environments. Studies of the functions of gp96 in these conditions by creating novel genetic tools and animal models should help to more completely uncover the physiological roles of gp96 in immune responses.

8. ACKNOWLEDGEMENT

The authors' work cited in this paper is supported by a NIH grant (to Z.L.). H.Z. and M.C. are supported by a NIH pre-doctoral training grant.

9. REFERENCES

1. Burnet F. M: The concept of immunological surveillance. *Prog Exp Tumor Res* 13, 1-27 (1970)

2. Gross L: Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Res 3, 323-326 (1943)

3. Baldwin R. W: Immunity to methylcholanthrene-induced tumors inn inbred rats following atrophy and regression of the implanted tumors. Br J Cancer 9, 652-657 (1955)

4. Prehn R. T & J. M. Main: Immunity to methylcholathrene-induced sarcomas. J Natl Cancer Inst 18, 769-778 (1957)

5. Klein G, H. O. Sjogren, E. Klein & K. E. Hellstrom: Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res* 20, 1561-1572 (1960)

6. Old L. J., E. A. Boyse, D. A. Clarke & E. A. Carswell: Antigenic properties of chemically induced tumors. *Ann NY Acad Sci* 101, 80-106 (1962)

7. Ullrich S. J, E. A. Robinson, L. W. Law, M. Willingham & E. Appella: A mouse tumor-specific transplantation antigen is a heat shock-related protein. *Proc Natl Acad Sci U S A* 83, 3121-3125 (1986)

8. Srivastava, P. K. & M. R. Das: The serologically unique cell surface antigen of Zajdela ascitic hepatoma is also its tumor-associated transplantation antigen. *Int J Cancer* 33, 417-422 (1984)

9. Srivastava P. K., A. B. DeLeo & L. J. Old: Tumor rejection antigens of chemically induced sarcomas of inbred mice. *Proc Natl Acad Sci U S A* 83, 3407-3411 (1986)

10. Palladino M. A. Jr., P. K. Srivastava, H. F. Oettgen & A. B. DeLeo: Expression of a shared tumor-specific antigen by two chemically induced BALB/c sarcomas. *Cancer Res* 47, 5074-5079 (1987)

11. Udono H & P. K. Srivastava: Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 178, 1391-1396 (1993)

12. Basu S & P. K. Srivastava: Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. *J Exp Med* 189, 797-802 (1999)

13. Wang X. Y, L. Kazim, E. A. Repasky & J. R. Subjeck: Characterization of heat shock protein 110 and glucose-regulated protein 170 as cancer vaccines and the effect of fever-range hyperthermia on vaccine activity. *J Immunol* 166, 490-497 (2001)

14. Lindquist S & E. A. Craig: The heat-shock proteins. *Annu Rev Genet* 22, 631-677 (1988)

15. Maki R. G, R. L. Eddy, Jr., M. Byers, T. B. Shows & P. K. Srivastava: Mapping of the genes for human endoplasmic reticular heat shock protein gp96/grp94. *Somat Cell Mol Genet* 19, 73-81 (1993)

16. Csermely P, T. Schnaider, C. Soti, Z. Prohaszka & G. Nardai: The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacol Ther* 79, 129-168 (1998)

17. Argon Y & B. B. Simen: GRP94, an ER chaperone with protein and peptide binding properties. *Semin Cell Dev Biol* 10, 495-505 (1999)

18. Srivastava P. K, Y. T. Chen & L. J. Old: 5'-structural analysis of genes encoding polymorphic antigens of chemically induced tumors. *Proc Natl Acad Sci U S A* 84, 3807-3811 (1987)

19. Mazzarella R. A & M. Green: ERp99, an abundant, conserved glycoprotein of the endoplasmic reticulum, is homologous to the 90-kDa heat shock protein (hsp90) and the 94-kDa glucose regulated protein (GRP94). *J Biol Chem* 262, 8875-8883 (1987)

20. Altmeyer A, R. G. Maki, A. M. Feldweg, M. Heike, V. P. Protopopov, S. K. Masur & P. K. Srivastava: Tumor-specific cell surface expression of the-KDEL containing, endoplasmic reticular heat shock protein gp96. *Int J Cancer* 69, 340-349 (1996)

21. Kozutsumi Y, M. Segal, K. Normington, M. J. Gething & J. Sambrook: The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* 332, 462-464 (1988)

22. Li Z. & P. K. Srivastava: Tumor rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation. *Embo J* 12, 3143-3151 (1993)

23. Srivastava P. K. & L. J. Old: Individually distinct transplantation antigens of chemically induced mouse tumors. *Immunol Today* 9, 78-83 (1988)

24. Srivastava P. K. & R. G. Maki: Stress-induced proteins in immune response to cancer. *Curr Top Microbiol Immunol* 167, 109-123 (1991)

25. Srivastava P. K. & M. Heike: Tumor-specific immunogenicity of stress-induced proteins: convergence of two evolutionary pathways of antigen presentation? *Semin Immunol* 3, 57-64 (1991)

26. Townsend A. R, F. M. Gotch & J. Davey: Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell* 42, 457-467 (1985)

27. Townsend A. & H. Bodmer: Antigen recognition by class I-restricted T lymphocytes. *Annu Rev Immunol* 7, 601-624 (1989)

28. Srivastava P. K, H. Udono, N. E. Blachere & Z. Li: Heat shock proteins transfer peptides during antigen processing and CTL priming. *Immunogenetics* 39, 93-98 (1994)

29. Li Z. & P. K. Srivastava: A critical contemplation on the role of heat shock proteins in transfer of antigenic peptides during antigen presentation. *Behring Inst Mitt*, 37-47 (1994)

30. Srivastava P. K, A. Menoret, S. Basu, R. J. Binder & K. L. McQuade: Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. *Immunity* 8, 657-665 (1998)

31. Schild H. & H. G. Rammensee: gp96--the immune system's Swiss army knife. *Nat Immunol* 1, 100-101 (2000)

32. Li Z, A. Menoret & P. K. Srivastava: Roles of heat shock proteins in antigen presentation and cross-presentation. Curr Opin Immunol 14, 45-51 (2002)

33. Multhoff G, C. Botzler, L. Jennen, J. Schmidt, J. Ellwart & R. Issels: Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. *J Immunol* 158, 4341-4350 (1997)

34. Multhoff G, C. Botzler, M. Wiesnet, G. Eissner & R. Issels: CD3- large granular lymphocytes recognize a heat-inducible immunogenic determinant associated with the 72-kD heat shock protein on human sarcoma cells. *Blood* 86, 1374-1382 (1995)

35. Multhoff G, C. Botzler, M. Wiesnet, E. Muller, T. Meier, W. Wilmanns & R. D. Issels: A stress-inducible 72-kDa heat-shock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. *Int J Cancer* 61, 272-279 (1995)

36. Kaur I, S. D. Voss, R. S. Gupta, K. Schell, P. Fisch & P. M. Sondel: Human peripheral gamma delta T cells recognize hsp60 molecules on Daudi Burkitt's lymphoma cells. *J Immunol* 150, 2046-2055 (1993)

37. Wei Y, X. Zhao, Y. Kariya, H. Fukata, K. Teshigawara & A. Uchida: Induction of autologous tumor killing by heat treatment of fresh human tumor cells: involvement of gamma delta T cells and heat shock protein 70. *Cancer Res* 56, 1104-1110 (1996)

38. Tamura Y, N. Tsuboi, N. Sato & K. Kikuchi: 70 kDa heat shock cognate protein is a transformation-associated antigen and a possible target for the host's anti-tumor immunity. *J Immunol* 151, 5516-5524 (1993)

39. Kishi A, T. Ichinohe, I. Hirai, K. Kamiguchi, Y. Tamura, M. Kinebuchi, T. Torigoe, S. Ichimiya, N. Kondo, K. Ishitani, T. Yoshikawa, M. Kondo, A. Matsuura & N. Sato: The cell surface-expressed HSC70-like molecule preferentially reacts with the rat T-cell receptor Vdelta6 family. *Immunogenetics* 53, 401-409 (2001)

40. Wiest D. L, A. Bhandoola, J. Punt, G. Kreibich, D. McKean & A. Singer: Incomplete endoplasmic reticulum (ER) retention in immature thymocytes as revealed by surface expression of "ER-resident" molecular chaperones. *Proc Natl Acad Sci U S A* 94, 1884-1889 (1997)

41. Robert J, A. Menoret & N. Cohen: Cell surface expression of the endoplasmic reticular heat shock protein gp96 is phylogenetically conserved. *J Immunol* 163, 4133-4139 (1999)

42. Singh-Jasuja H, R. E. Toes, P. Spee, C. Munz, N. Hilf, S. P. Schoenberger, P. Ricciardi-Castagnoli, J. Neefjes, H. G. Rammensee, D. Arnold-Schild & H. Schild: Cross-presentation of glycoprotein 96-associated antigens on major histocompatibility complex class I molecules requires receptor-mediated endocytosis. *J Exp Med* 191, 1965-1974 (2000)

43. Linderoth N. A, A. Popowicz & S. Sastry: Identification of the peptide-binding site in the heat shock chaperone/tumor rejection antigen gp96 (Grp94). *J Biol Chem* 275, 5472-5477

(2000)

44. Ishii T, H. Udono, T. Yamano, H. Ohta, A. Uenaka, T. Ono, A. Hizuta, N. Tanaka, P. K. Srivastava & E. Nakayama: Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. *J Immunol* 162, 1303-1309 (1999)

45. Menoret A, P. Peng & P. K. Srivastava: Association of peptides with heat shock protein gp96 occurs *in vivo* and not after cell lysis. *Biochem Biophys Res Commun* 262, 813-818 (1999)

46. Booth C. & G. L. Koch: Perturbation of cellular calcium induces secretion of luminal ER proteins. *Cell* 59, 729-737 (1989)

47. Menoret A, Z. Li, M. L. Niswonger, A. Altmeyer & P. K. Srivastava: An Endoplasmic Reticulum Protein Implicated in Chaperoning Peptides to Major Histocompatibility of Class I Is an Aminopeptidase. *J Biol Chem* 276, 33313-33318 (2001)

48. Binder R. J, D. K. Han & P. K. Srivastava: CD91: a receptor for heat shock protein gp96. *Nat Immunol* 1, 151-155 (2000)

49. Basu S, R. J. Binder, T. Ramalingam & P. K. Srivastava: CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 14, 303-313 (2001)

50. Panjwanji N. N, L. P. M. Febbraio & P. K. Srivastava: The CD36 scavenger receptor as a receptor for gp96. Cell Stress & Chaperones 5, 391 (2000)

51. Flynn G. C, T. G. Chappell & J. E. Rothman: Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* 245, 385-390 (1989)

52. Flynn G.C, J. Pohl, M. T. Flocco & J. E. Rothman: Peptide-binding specificity of the molecular chaperone Bip. Nature 353, 726-730 (1991)

53. Blond-Elguindi S, S. E. Cwirla, W. J. Dower, R. J. Lipshutz, S. R. Sprang, J. F. Sambrook & M. J. Gething: Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell* 75, 717-728 (1993)

54. Zhu X, X. Zhao, W. F. Burkholder, A. Gragerov, C. M. Ogata, M. E. Gottesman & W. A. Hendrickson: Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* 272, 1606-1614 (1996)

55. Heike M, N. E. Blachere, T. Wolfel, K. H. Meyer zum Buschenfelde, S. Storkel & P. K. Srivastava: Membranes activate tumor- and virus-specific precursor cytotoxic T lymphocytes *in vivo* and stimulate tumor-specific T lymphocytes *in vitro*: implications for vaccination. *J Immunother Emphasis Tumor Immunol* 15, 165-174 (1994)

56. Nieland T. J, M. C. Tan, M. Monne-van Muijen, F. Koning, A. M. Kruisbeek & G. M. van Bleek: Isolation of an immunodominant viral peptide that is endogenously bound to the stress protein GP96/GRP94. *Proc Natl Acad Sci U S A* 93, 6135-6139 (1996)

57. Arnold D, S. Faath, H. Rammensee & H. Schild: Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with the heat shock protein gp96. *J Exp Med* 182, 885-889 (1995)

58. Blachere N. E, H. Udono, S. Janetzki, Z. Li, M. Heike & P. K. Srivastava: Heat shock protein vaccines against cancer. *J Immunother* 14, 352-356 (1993)

59. Zugel U, A. M. Sponaas, J. Neckermann, B. Schoel & S. H. Kaufmann: gp96-peptide vaccination of mice against intracellular bacteria. *Infect Immun* 69, 4164-4167 (2001)

60. Breloer M, T. Marti, B. Fleischer & A. von Bonin: Isolation of processed, H-2Kb-binding ovalbumin-derived peptides associated with the stress proteins HSP70 and gp96. *Eur J Immunol* 28, 1016-1021 (1998)

61. Spee P, J. Subjeck & J. Neefjes: Identification of novel peptide binding proteins in the endoplasmic reticulum: ERp72, calnexin, and grp170. *Biochemistry* 38, 10559-10566 (1999)

62. Lammert E, D. Arnold, M. Nijenhuis, F. Momburg, G. J. Hammerling, J. Brunner, S. Stevanovic, H. G. Rammensee & H. Schild: The endoplasmic reticulum-resident stress protein gp96 binds peptides translocated by TAP. *Eur J Immunol* 27, 923-927 (1997)

63. Blachere N. E, Z. Li, R. Y. Chandawarkar, R. Suto, N. S. Jaikaria, S. Basu, H. Udono & P. K. Srivastava: Heat shock protein-peptide complexes, reconstituted *in vitro*, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J Exp Med* 186, 1315-1322 (1997)

64. Wearsch P. A. & C. V. Nicchitta: Endoplasmic reticulum chaperone GRP94 subunit assembly is regulated through a defined oligomerization domain. *Biochemistry* 35, 16760-16769 (1996)

65. Wearsch P. A, L. Voglino & C. V. Nicchitta: Structural transitions accompanying the activation of peptide binding to the endoplasmic reticulum Hsp90 chaperone GRP94. *Biochemistry* 37, 5709-5719 (1998)

66. Linderoth L. A, M. N. Simon, J. F. Hainfeld & S. Sastry: Binding of Antigenic Peptide to the Endoplasmic Reticulum-resident Protein gp96/GRP94 Heat Shock Chaperone Occurs in Higher Order Complexes. Essential role of some aromatic amino acid residues in the peptide-binding site. *J Biol Chem* 276, 11049-11054 (2001)

67. Prodromou C, S. M. Roe, R. O'Brien, J. E. Ladbury, P. W. Piper & L. H. Pearl: Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 90, 65-75 (1997)

68. Stebbins C. E, A. A. Russo, C. Schneider, N. Rosen, F. U. Hartl & N. P. Pavletich: Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* 89, 239-250 (1997)

69. Whitesell L, E. G. Mimnaugh, B. De Costa, C. E. Myers & L. M. Neckers: Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone

ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 91, 8324-8328 (1994)

70. Obermann W. M, H. Sondermann, A. A. Russo, N. P. Pavletich & F. U. Hartl: *In vivo* function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *J Cell Biol* 143, 901-910 (1998)

71. Csermely P, Y. Miyata, T. Schnaider & I. Yahara: Autophosphorylation of grp94 (endoplasmin). *J Biol Chem* 270, 6381-6388 (1995)

72. Wearsch P. A. & C. V. Nicchitta: Interaction of endoplasmic reticulum chaperone GRP94 with peptide substrates is adenine nucleotide-independent. *J Biol Chem* 272, 5152-5156 (1997)

73. Rosser M. F. & C. V. Nicchitta: Ligand interactions in the adenosine nucleotide-binding domain of the Hsp90 chaperone, GRP94. I. Evidence for allosteric regulation of ligand binding. *J Biol Chem* 275, 22798-22805 (2000)

74. Randow F. & B. Seed: Endoplasmic reticulum chaperone gp96 is required for innate immunity but not cell viability. *Nature cell biology* 3, 891-896 (2001)

75. Koopmann J. O, J. Albring, E. Huter, N. Bulbuc, P. Spee, J. Neefjes, G. J. Hammerling & F. Momburg: Export of antigenic peptides from the endoplasmic reticulum intersects with retrograde protein translocation through the Sec61p channel. *Immunity* 13, 117-127 (2000)

76. Serwold T, S. Gaw & N. Shastri: ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. *Nat Immunol* 2, 644-651 (2001)

77. Stoltze L, M. Schirle, G. Schwarz, C. Schroter, M. W. Thompson, L. B. Hersh, H. Kalbacher, S. Stevanovic, H. G. Rammensee & H. Schild: Two new proteases in the MHC class I processing pathway. *Nat Immunol* 1, 413-418 (2000)

78. Brouwenstijn N, T. Serwold & N. Shastri: MHC class I molecules can direct proteolytic cleavage of antigenic precursors in the endoplasmic reticulum. *Immunity* 15, 95-104 (2001)

79. Fruci D, G. Niedermann, R. H. Butler & P. M. van Endert: Efficient MHC class I-independent amino-terminal trimming of epitope precursor peptides in the endoplasmic reticulum. *Immunity* 15, 467-476 (2001)

80. Lobigs M, G. Chelvanayagam & A. Mullbacher: Proteolytic processing of peptides in the lumen of the endoplasmic reticulum for antigen presentation by major histocompatibility class I. *Eur J Immunol* 30, 1496-1506 (2000)

81. Kappler J. W, N. Roehm & P. Marrack: T cell tolerance by clonal elimination in the thymus. *Cell* 49, 273-280 (1987)

82. Miller J. F. & G. Morahan: Peripheral T cell tolerance. *Annu Rev Immunol* 10, 51-69 (1992)

83. Janeway C. A, Jr: Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54, 1-13 (1989)

84. Matzinger P: Tolerance, danger, and the extended family. *Annu Rev Immunol* 12, 991-1045 (1994)

85. Banchereau J. & R. M. Steinman: Dendritic cells and the control of immunity. *Nature* 392, 245-252 (1998)

86. Udono H, D. L. Levey & P. K. Srivastava: Cellular requirements for tumor-specific immunity elicited by heat shock proteins: tumor rejection antigen gp96 primes CD8+ T cells *in vivo*. *Proc Natl Acad Sci U S A* 91, 3077-3081 (1994)

87. Suto R. & P. K. Srivastava: A mechanism for the specific immunogenicity of heat shock protein- chaperoned peptides. *Science* 269, 1585-1588 (1995)

88. Arnold-Schild D, D. Hanau, D. Spehner, C. Schmid, H. G. Rammensee, H. de la Salle & H. Schild: Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J Immunol* 162, 3757-3760 (1999)

89. Singh-Jasuja H, H. U. Scherer, N. Hilf, D. Arnold-Schild, H. G. Rammensee, R. E. Toes & H. Schild: The heat shock protein gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur J Immunol* 30, 2211-2215 (2000)

90. Wassenberg J. J, C. Dezfulian & C. V. Nicchitta: Receptor mediated and fluid phase pathways for internalization of the ER Hsp90 chaperone GRP94 in murine macrophages. *J Cell Sci* 112, 2167-2175 (1999)

91. Binder R. J., M. L. Harris, A. Menoret & P. K. Srivastava: Saturation, competition, and specificity in interaction of heat shock proteins (hsp) gp96, hsp90, and hsp70 with CD11b+ cells. *J Immunol* 165, 2582-2587 (2000)

92. De Smedt T, B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo & M. Moser: Regulation of dendritic cell numbers and maturation by lipopolysaccharide *in vivo*. *J Exp Med* 184, 1413-1424 (1996)

93. Hartmann G, G. J. Weiner & A. M. Krieg: CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc Natl Acad Sci U S A* 96, 9305-9310 (1999)

94. Krieg A. M: The role of CpG motifs in innate immunity. *Curr Opin Immunol* 12, 35-43 (2000)

95. Roake J. A, A. S. Rao, P. J. Morris, C. P. Larsen, D. F. Hankins & J. M. Austyn: Dendritic cell loss from nonlymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J Exp Med* 181, 2237-2247 (1995)

96. Gallucci S, M. Lolkema & P. Matzinger: Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 5, 1249-1255 (1999)

97. Sauter B, M. L. Albert, L. Francisco, M. Larsson, S. Somersan & N. Bhardwaj: Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 191, 423-434 (2000)

98. Basu S, R. J. Binder, R. Suto, K. M. Anderson & P. K. Srivastava: Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. *Int Immunol* 12, 1539-1546 (2000)

99. Yamazaki K, T. Nguyen & E. R. Podack: Cutting edge: tumor secreted heat shock-fusion protein elicits CD8 cells for rejection. *J Immunol* 163, 5178-5182 (1999)

100. Zheng H, J. Dai, D. Stoilova & Z. Li: Cell surface expression of heat shock protein gp96 induced dendritic cell maturation and anti-tumor immunity. J Immunol 167, 6731-6735 (2001)

101. Dieu M. C, B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque & C. Caux: Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* 188, 373-386 (1998)

102. Sallusto F, B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp & A. Lanzavecchia: Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol* 29, 1617-1625 (1999)

103. Yanagihara S, E. Komura, J. Nagafune, H. Watarai & Y. Yamaguchi: EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. *J Immunol* 161, 3096-3102 (1998)

104. Ngo V. N, H. L. Tang & J. G. Cyster: Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J Exp Med* 188, 181-191 (1998)

105. Sallusto F, P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C. R. Mackay, S. Qin & A. Lanzavecchia: Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* 28, 2760-2769 (1998)

106. Sozzani S, P. Allavena, G. D'Amico, W. Luini, G. Bianchi, M. Kataura, T. Imai, O. Yoshie, R. Bonecchi & A. Mantovani: Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J Immunol* 161, 1083-1086 (1998)

107. Binder R. J, K. M. Anderson, S. Basu & P. K. Srivastava: Cutting edge: heat shock protein gp96 induces maturation and migration of CD11c+ cells *in vivo*. *J Immunol* 165, 6029-6035 (2000)

108. Yewdell J. W. & J. R. Bennink: The binary logic of antigen processing and presentation to T cells. *Cell* 62, 203-206 (1990)

109. den Haan J. M. & M. J. Bevan: Antigen presentation to CD8+ T cells: cross-priming in infectious diseases. *Curr Opin Immunol* 13, 437-441 (2001)

110. Huang A. Y, A. T. Bruce, D. M. Pardoll & H. I. Levitsky: *In vivo* cross-priming of MHC class I-restricted antigens requires the TAP transporter. *Immunity* 4, 349-355 (1996)

111. den Haan J. M, S. M. Lehar & M. J. Bevan: CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells *in vivo*. *J Exp Med* 192, 1685-1696 (2000)

112. Arnold D, C. Wahl, S. Faath, H. G. Rammensee & H. Schild: Influences of transporter associated with antigen processing (TAP) on the repertoire of peptides associated with the endoplasmic reticulum- resident stress protein gp96. *J Exp Med* 186, 461-466 (1997)

113. Matsutake T. & P. K. Srivastava: The immunoprotective MHC II epitope of a chemically induced tumor harbors a unique mutation in a ribosomal protein. *Proc Natl Acad Sci U S A* 98, 3992-3997 (2001)

114. Li M, G. M. Davey, R. M. Sutherland, C. Kurts, A. M. Lew, C. Hirst, F. R. Carbone & W. R. Heath: Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin *in vivo*. *J Immunol* 166, 6099-6103 (2001)

115. Chandawarkar R. Y, M. S. Wagh & P. K. Srivastava: The dual nature of specific immunological activity of tumor-derived gp96 preparations. *J Exp Med* 189, 1437-1442 (1999)

116. Navaratnam M, M. S. Deshpande, M. J. Hariharan, D. S. Zatechka, Jr. & S. Srikumaran: Heat shock protein-peptide complexes elicit cytotoxic T-lymphocyte and antibody responses specific for bovine herpesvirus 1. *Vaccine* 19, 1425-1434 (2001)

117. Menoret A, R. Y. Chandawarkar & P. K. Srivastava: Natural autoantibodies against heat-shock proteins hsp70 and gp96: implications for immunotherapy using heat-shock proteins. *Immunology* 101, 364-370 (2000)

118. Zhu Y, S. O'Neill, J. Saklatvala, L. Tassi & M. E. Mendelsohn: Phosphorylated HSP27 associates with the activation-dependent cytoskeleton in human platelets. *Blood* 84, 3715-3723 (1994)

119. Polanowska-Grabowska R, C. G. Simon, Jr., R. Falchetto, J. Shabanowitz, D. F. Hunt & A. R. Gear: Platelet adhesion to collagen under flow causes dissociation of a phosphoprotein complex of heat-shock proteins and protein phosphatase 1. *Blood* 90, 1516-1526 (1997)

120. Schubert U, L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell & J. R. Bennink: Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404, 770-774 (2000)

121. Turner G. C & A. Varshavsky: Detecting and measuring cotranslational protein degradation *in vivo*. *Science* 289, 2117-2120 (2000)

122. Reits E. A. J, J. C. Vos, M. Gromme & J. Neefjes: The major substrates for TAP *in vivo* are derived from newly synthesized proteins. Nature 404, 774-778 (2000)

123. Chen D. & M. J. Androlewicz: Heat shock protein 70 moderately enhances peptide binding and transport by the transporter associated with antigen processing. *Immunol Lett* 75,

143-148 (2001)

124. Anderson S. L, T. Shen, J. Lou, L. Xing, N. E. Blachere, P. K. Srivastava & B. Y. Rubin: The endoplasmic reticular heat shock protein gp96 is transcriptionally upregulated in interferon-treated cells. *J Exp Med* 180, 1565-1569 (1994)

125. Levy F, R. Gabathuler, R. Larsson & S. Kvist: ATP is required for *in vitro* assembly of MHC class I antigens but not for transfer of peptides across the ER membrane. *Cell* 67, 265-274 (1991)

126. Lammert E, D. Arnold, H. G. Rammensee & H. Schild: Expression levels of stress protein gp96 are not limiting for major histocompatibility complex class I-restricted antigen presentation. *Eur J Immunol* 26, 875-879 (1996)

127. Bevan M. J: Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* 143, 1283-1288 (1976)

128. Bevan M. J: Minor H antigens introduced on H-2 different stimulating cells cross- react at the cytotoxic T cell level during *in vivo* priming. *J Immunol* 117, 2233-2238 (1976)

129. Regnault A, D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli & S. Amigorena: Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 189, 371-380 (1999)

130. Schoenberger S. P, E. I. van der Voort, G. M. Krietemeijer, R. Offringa, C. J. Melief & R. E. Toes: Cross-priming of CTL responses *in vivo* does not require antigenic peptides in the endoplasmic reticulum of immunizing cells. *J Immunol* 161, 3808-3812 (1998)

131. Piccirillo C. A. & E. M. Shevach: Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol* 167, 1137-1140 (2001)

132. Chen Y, V. K. Kuchroo, J. Inobe, D. A. Hafler & H. L. Weiner: Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, 1237-1240 (1994)

133. Seddon B. & D. Mason: Regulatory T cells in the control of autoimmunity: the essential role of transforming growth factor beta and interleukin 4 in the prevention of autoimmune thyroiditis in rats by peripheral CD4(+)CD45RC- cells and CD4(+)CD8(-) thymocytes. *J Exp Med* 189, 279-288 (1999)

134. Zhang Z. X, L. Yang, K. J. Young, B. DuTemple & L. Zhang: Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat* Med 6, 782-789 (2000)

135. Bendelac A, M. N. Rivera, S. H. Park & J. H. Roark: Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 15, 535-562 (1997)

136. Hammond K. J, S. B. Pelikan, N. Y. Crowe, E. Randle-Barrett, T. Nakayama, M. Taniguchi, M. J. Smyth, I. R. van Driel, R. Scollay, A. G. Baxter & D. I. Godfrey: NKT cells are phenotypically and functionally diverse. *Eur J Immunol* 29, 3768-3781 (1999)

137. Apostolou I, A. Cumano, G. Gachelin & P. Kourilsky: Evidence for two subgroups of CD4-CD8- NKT cells with distinct TCR alpha beta repertoires and differential distribution in lymphoid tissues. *J Immunol* 165, 2481-2490 (2000)

138. Cui J, T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno & M. Taniguchi: Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278, 1623-1626 (1997)

139. Smyth M. J, K. Y. Thia, S. E. Street, E. Cretney, J. A. Trapani, M. Taniguchi, T. Kawano, S. B. Pelikan, N. Y. Crowe & D. I. Godfrey: Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* 191, 661-668 (2000)

140. Terabe M, S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D. D. Donaldson, D. P. Carbone, W. E. Paul & J. A. Berzofsky: NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* 1, 515-520 (2000)

141. Hammond K. J, L. D. Poulton, L. J. Palmisano, P. A. Silveira, D. I. Godfrey & A. G. Baxter: alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J Exp Med* 187, 1047-1056 (1998)

142. Carding S. R. & P. J. Egan: The importance of gamma delta T cells in the resolution of pathogen- induced inflammatory immune responses. *Immunol Rev* 173, 98-108 (2000)

143. Hanninen A. & L. C. Harrison: Gamma delta T cells as mediators of mucosal tolerance: the autoimmune diabetes model. *Immunol Rev* 173, 109-119 (2000)

144. Seo N, Y. Tokura, M. Takigawa & K. Egawa: Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells. *J Immunol* 163, 242-249 (1999)

145. Yewdell J. W: Not such a dismal science: the economics of protein synthesis, folding, degradation and antigen processing. *Trends Cell Biol* 11, 294-297 (2001)

146. Srivastava P. K: Endo-beta-D-glucuronidase (heparanase) activity of heat-shock protein/tumour rejection antigen gp96. *Biochem J* 301, 919 (1994)

147. Li L. J, X. Li, A. Ferrario, N. Rucker, E. S. Liu, S. Wong, C. J. Gomer & A. S. Lee: Establishment of a Chinese hamster ovary cell line that expresses grp78 antisense transcripts and suppresses A23187 induction of both GRP78 and GRP94. *J Cell Physiol* 153, 575-582 (1992)

148. Meng S. D, T. Gao, G. F. Gao & P. Tien: HBV-specific peptide associated with heat-shock protein gp96. *Lancet* 357, 528-529 (2001)

149. Udono H. & P. K. Srivastava: Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J Immunol* 152, 5398-5403 (1994)

150. Tamura Y, P. Peng, K. Liu, M. Daou & P. K. Srivastava: Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* 278, 117-120 (1997)

151. Janetzki S, N. E. Blachere & P. K. Srivastava: Generation of tumor-specific cytotoxic T lymphocytes and memory T cells by immunization with tumor-derived heat shock protein gp96. *J Immunother* 21, 269-276 (1998)

152. Yedavelli S. P, L. Guo, M. E. Daou, P. K. Srivastava, A. Mittelman & R. K. Tiwari: Preventive and therapeutic effect of tumor derived heat shock protein, gp96, in an experimental prostate cancer model. *Int J Mol Med* 4, 243-248 (1999)

153. Graner M, A. Raymond, D. Romney, L. He, L. Whitesell & E. Katsanis: Immunoprotective activities of multiple chaperone proteins isolated from murine B-cell leukemia/lymphoma. *Clin Cancer Res* 6, 909-915 (2000)

154. Robert J, A. Menoret, S. Basu, N. Cohen & P. R. Srivastava: Phylogenetic conservation of the molecular and immunological properties of the chaperones gp96 and hsp70. *Eur J Immunol* 31, 186-195 (2001)

155. Asea A, S. K. Kraeft, E. A. Kurt-Jones, M. A. Stevenson, L. B. Chen, R. W. Finberg, G. C. Koo & S. K. Calderwood: HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6, 435-442 (2000)

156. Kuppner M. C, R. Gastpar, S. Gelwer, E. Nossner, O. Ochmann, A. Scharner & R. D. Issels: The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. *Eur J Immunol* 31, 1602-1609 (2001)

157. Moroi Y, M. Mayhew, J. Trcka, M. H. Hoe, Y. Takechi, F. U. Hartl, J. E. Rothman & A. N. Houghton: Induction of cellular immunity by immunization with novel hybrid peptides complexed to heat shock protein 70. *Proc Natl Acad Sci U S A* 97, 3485-3490 (2000)

158. Breloer M, B. Dorner, S. H. More, T. Roderian, B. Fleischer & A. von Bonin: Heat shock proteins as "danger signals": eukaryotic Hsp60 enhances and accelerates antigen-specific IFN-gamma production in T cells. *Eur J Immunol* 31, 2051-2059 (2001)

159. Kol A, A. H. Lichtman, R. W. Finberg, P. Libby & E. A. Kurt-Jones: Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J Immunol* 164, 13-17 (2000)

160. Munro S. & H. R. Pelham: A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899-907 (1987)

161. Realini C, S. W. Rogers & M. Rechsteiner: KEKE motifs. Proposed roles in protein-protein association and presentation of peptides by MHC class I receptors. FEBS Lett 348, 109-113 (1994)

162. Maruya M, M. Sameshima, T. Nemoto, & I. Yahara: Monomer arrangement in HSP90 dimer as determined by decoration with N and C-terminal region specific antibodies. *J Mol Biol* 285, 903-907 (1999)

163. Nemoto T, Y. Ohara-Nemoto, M. Ota, T. Takagi & K. Yokoyama: Mechanism of dimer formation of the 90-kDa heat-shock protein. *Eur J Biochem* 233, 1-8 (1995)

164. Minami Y, H. Kawasaki, M. Minami, N. Tanahashi, K. Tanaka & I. Yahara: A critical role for the proteasome activator PA28 in the Hsp90-dependent protein refolding. *J Biol Chem 275,* 9055-9061 (2000)

Key words: Heat shock protein, gp96, APC, MHC, antigen presentation, cross priming, CTL, tumor antigens, cancer vaccine

Send correspondence to: Zihai Li, M.D., Ph.D., Center for Immunotherapy of Cancer and Infectious Diseases, MC 1601, University of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06030-1601, U.S.A, Tel:860-679-7979, Fax: 860-679-1265, E-mail: zli@up.uchc.edu

09/736146

* *

```
=> s apoptosis and (stromal cells)

           370 APOPTOSIS
           929 STROMAL
        165497 CELLS
           350 STROMAL CELLS
                 (STROMAL(W)CELLS)
L1          19 APOPTOSIS AND (STROMAL CELLS)

=> s l1 and bone marrow cells

         30482 BONE
          5826 MARROW
        165497 CELLS
          1095 BONE MARROW CELLS
                 (BONE(W)MARROW(W)CELLS)
L2           6 L1 AND BONE MARROW CELLS

=> d l2 1-6 bib ab
```

US PAT NO: 5,733,541 [IMAGE AVAILABLE] L2: 1 of 6
DATE ISSUED: Mar. 31, 1998
TITLE: Hematopoietic cells: compositions and methods
INVENTOR: Russell S. Taichman, Ann Arbor, MI
Stephen G. Emerson, Wayne, PA
ASSIGNEE: The Regent of the University of Michigan, Ann Arbor, MI (U.S. corp.)
APPL-NO: 08/426,792
DATE FILED: Apr. 21, 1995
ART-UNIT: 184
PRIM-EXMR: Jasemine C. Chambers
ASST-EXMR: Deborah J. R. Clark
LEGAL-REP: Arnold, White & Durkee

US PAT NO: 5,733,541 [IMAGE AVAILABLE] L2: 1 of 6

ABSTRACT:
Processes, compositions and uses of hematopoietic cells are disclosed. Hematopoietic cells are cells which can differentiate into mature blood cells when co-cultured with osteoblasts. Specifically, a process for propagating and maintaining the immature morphology of a hematopoietic cell by co-culturing with osteoblasts is disclosed. The osteoblasts provide cytokines and/or a microenvironment which propagates and maintains the immature morphology of a hematopoietic cell. Hematopoietic cells are useful in the treatment of certain blood related disorders and are useful for treatment of patients in need of hematopoietic cells.

US PAT NO: 5,705,615 [IMAGE AVAILABLE] L2: 2 of 6
DATE ISSUED: Jan. 6, 1998
TITLE: Antibodies specific for HT.sub.m4
INVENTOR: Bing Lim, Dorchester, MA
Chaker N. Adra, Boston, MA

Jean-Michel Lelias, Columbus, OH
ASSIGNEE: Beth Israel Deaconess Medical Center, Boston, MA (U.S. corp.)
APPL-NO: 08/707,340
DATE FILED: Sep. 3, 1996
ART-UNIT: 186
PRIM-EXMR: Christina Y. Chan
ASST-EXMR: Emma Cech
LEGAL-REP: Hamilton, Brook, Smith & Reynolds, P.C.

US PAT NO: 5,705,615 [IMAGE AVAILABLE] L2: 2 of 6

ABSTRACT:
The invention relates to a recombinant DNA molecule which encodes a HT.sub.m4 protein, a transformed host cell which has been stably transfected with a DNA molecule which encodes a HT.sub.m4 protein and a recombinant HT.sub.m4 protein. The invention also relates to a method for detecting the presence of a hereditary atopy.

US PAT NO: 5,658,761 [IMAGE AVAILABLE] L2: 3 of 6
DATE ISSUED: Aug. 19, 1997
TITLE: Stromal cell lines from human bone marrow and their use
INVENTOR: Karin Thalmeier, Munchen, Federal Republic of Germany
Peter Dormer, Gilching, Federal Republic of Germany
ASSIGNEE: Gsf-Forschungszentrum fur Umwelt und Gesundheit GmbH, OberscheleiBheim, Federal Republic of Germany (foreign corp.)
APPL-NO: 08/584,425
DATE FILED: Jan. 11, 1996
ART-UNIT: 185
PRIM-EXMR: James Ketter
LEGAL-REP: Nikaido, Marmelstein, Murray & Oram

US PAT NO: 5,658,761 [IMAGE AVAILABLE] L2: 3 of 6

ABSTRACT:
A human bone marrow stromal cell line, which is characterized in that the cells of the cell line, after irradiation which results in the growth being arrested, remain adherent, is suitable for use as a feeder layer for supporting the proliferation of blood cells.

US PAT NO: 5,625,122 [IMAGE AVAILABLE] L2: 4 of 6
DATE ISSUED: Apr. 29, 1997
TITLE: Mouse having a disrupted lck gene
INVENTOR: Tak W. Mak, Toronto, Canada
ASSIGNEE: The Ontario Cancer Institute, Toronto, Canada (foreign corp.)
APPL-NO: 08/145,043
DATE FILED: Nov. 3, 1993
ART-UNIT: 184
PRIM-EXMR: Jacqueline M. Stone
ASST-EXMR: Deborah Crouch
LEGAL-REP: Marshall, O'Toole, Gerstein, Murray & Borun

US PAT NO: 5,625,122 [IMAGE AVAILABLE] L2: 4 of 6

ABSTRACT:
A mutant non-human mammal lacking expression of the lymphocyte-specific tyrosine kinase p56.sup.lck. Lck deficient mice possess few peripheral T lymphocytes and a pronounced thymic atrophy. The remaining thymus contains immature thymocytes surrounded by a perturbed thymic microenvironment. p56.sup.lck appears to play a crucial role in early thymocyte differentiation.

US PAT NO: 5,612,211 [IMAGE AVAILABLE] L2: 5 of 6
DATE ISSUED: Mar. 18, 1997
TITLE: Stimulation, production and culturing of hematopoietic progenitor cells by fibroblast growth factors
INVENTOR: Elaine L. Wilson, New York, NY
Janice Gabrilove, New York, NY
ASSIGNEE: New York University, New York, NY (U.S. corp.)
Sloan-Kettering Institute For Cancer Research, New York, NY (U.S. corp.)
APPL-NO: 08/076,875
DATE FILED: Jun. 15, 1993
ART-UNIT: 181
PRIM-EXMR: Christina Y. Chan
ASST-EXMR: David Lukton
LEGAL-REP: Browdy and Neimark

US PAT NO: 5,612,211 [IMAGE AVAILABLE] L2: 5 of 6

ABSTRACT:
Fibroblast growth factors are used in vivo, in situ and in vitro to stimulate stem cells, hemopoiesis, the immune system, transplant donor cells, culture and/or engraftment, wherein the use of fibroblast growth factors is disclosed for the stimulation of stem cells or hemopoietic cells, supporting cells and their progeny, in vitro, in situ and in vivo, as well as corresponding engrafting sites in vivo.

US PAT NO: 5,521,067 [IMAGE AVAILABLE] L2: 6 of 6
DATE ISSUED: May 28, 1996
TITLE: Bone marrow cell adhesion molecules and process for detecting adherence between cell adhesion molecules and cells generally
INVENTOR: Beerelli Seshi, Fairport, NY
ASSIGNEE: University of Rochester, Rochester, NY (U.S. corp.)
APPL-NO: 08/158,936
DATE FILED: Nov. 24, 1993
ART-UNIT: 182
PRIM-EXMR: David Saunders
LEGAL-REP: Nixon, Hargrave, Devans & Doyle

US PAT NO: 5,521,067 [IMAGE AVAILABLE] L2: 6 of 6

ABSTRACT:
The present invention relates to proteins associated with human bone marrow cell membranes for adhering hematopoietic cells to human bone marrow cell membranes. These proteins are soluble in lithium dodecyl sulfate but insoluble in 2% nonaethylene glycol octylphenol ether (e.g.,

2% Triton.RTM. X-100) solution. These proteins and antibodies raised against them are useful in the treatment and diagnosis of blood disorders. The DNA molecules encoding these proteins have use in gene therapy regimes. Also disclosed is a method for detecting binding between cell adhesion membrane proteins and cells having a potential to be bound to such proteins.

=> s L1 and antibod?

```
         28409 ANTIBOD?
L3          18 L1 AND ANTIBOD?
```

=> d l3 1-18 bib ab

US PAT NO: 5,733,541 [IMAGE AVAILABLE] L3: 1 of 18
DATE ISSUED: Mar. 31, 1998
TITLE: Hematopoietic cells: compositions and methods
INVENTOR: Russell S. Taichman, Ann Arbor, MI
Stephen G. Emerson, Wayne, PA
ASSIGNEE: The Regent of the University of Michigan, Ann Arbor, MI (U.S. corp.)
APPL-NO: 08/426,792
DATE FILED: Apr. 21, 1995
ART-UNIT: 184
PRIM-EXMR: Jasemine C. Chambers
ASST-EXMR: Deborah J. R. Clark
LEGAL-REP: Arnold, White & Durkee

US PAT NO: 5,733,541 [IMAGE AVAILABLE] L3: 1 of 18

ABSTRACT:
Processes, compositions and uses of hematopoietic cells are disclosed. Hematopoietic cells are cells which can differentiate into mature blood cells when co-cultured with osteoblasts. Specifically, a process for propagating and maintaining the immature morphology of a hematopoietic cell by co-culturing with osteoblasts is disclosed. The osteoblasts provide cytokines and/or a microenvironment which propagates and maintains the immature morphology of a hematopoietic cell. Hematopoietic cells are useful in the treatment of certain blood related disorders and are useful for treatment of patients in need of hematopoietic cells.

US PAT NO: 5,728,819 [IMAGE AVAILABLE] L3: 2 of 18
DATE ISSUED: Mar. 17, 1998
TITLE: Secreted proteins and polynucleotides encoding them
INVENTOR: Kenneth Jacobs, Newton, MA
John M. McCoy, Reading, MA
Edward R. LaVallie, Tewksbury, MA
Lisa A. Racie, Acton, MA
David Merberg, Acton, MA
Maurice Treacy, Chestnut Hill, MA
Cheryl Evans, Brookline, MA
Vikki Spaulding, Billerica, MA
ASSIGNEE: Genetics Institute, Inc., Cambridge, MA (U.S. corp.)
APPL-NO: 08/691,641

DATE FILED: Aug. 2, 1996
ART-UNIT: 182
PRIM-EXMR: Stephen Walsh
ASST-EXMR: Claire M. Kaufman
LEGAL-REP: Scott A. Brown, Thomas J. DesRosier

US PAT NO: 5,728,819 [IMAGE AVAILABLE] L3: 2 of 18

ABSTRACT:
Novel polynucleotides and the proteins encoded thereby are disclosed.

US PAT NO: 5,723,315 [IMAGE AVAILABLE] L3: 3 of 18
DATE ISSUED: Mar. 3, 1998
TITLE: Secreted proteins and polynucleotides encoding them
INVENTOR: Kenneth Jacobs, Newton, MA
John M. McCoy, Reading, MA
Edward R. LaVallie, Tewksbury, MA
Lisa A. Racie, Acton, MA
David Merberg, Acton, MA
Maurice Treacy, Chestnut Hill, MA
Vikki Spaulding, Billerica, MA
ASSIGNEE: Genetics Institute, Inc., Cambridge, MA (U.S. corp.)
APPL-NO: 08/702,344
DATE FILED: Aug. 23, 1996
ART-UNIT: 182
PRIM-EXMR: Stephen Walsh
ASST-EXMR: Claire M. Kaufman
LEGAL-REP: Scott A. Brown, Suzanne A. Sprunger, Thomas J. DesRosier

US PAT NO: 5,723,315 [IMAGE AVAILABLE] L3: 3 of 18

ABSTRACT:
Novel polynucleotides and the proteins encoded thereby are disclosed.

US PAT NO: 5,708,157 [IMAGE AVAILABLE] L3: 4 of 18
DATE ISSUED: Jan. 13, 1998
TITLE: Secreted proteins and polynucleotides encoding them
INVENTOR: Kenneth Jacobs, Newton, MA
John M. McCoy, Reading, MA
Edward R. LaVallie, Tewskbury, MA
Lisa A. Racie, Acton, MA
David Merberg, Acton, MA
Maurice Treacy, Chestnut Hill, MA
Cheryl Evans, Brookline, MA
Vikki Spaulding, Billerica, MA
ASSIGNEE: Genetics Institute, Inc., Cambridge, MA (U.S. corp.)
APPL-NO: 08/686,878
DATE FILED: Jul. 26, 1996
ART-UNIT: 182
PRIM-EXMR: Stephen Walsh
ASST-EXMR: Claire M. Kaufman
LEGAL-REP: Scott A. Brown, Suzanne A. Sprunger, Thomas J. DesRosier

US PAT NO: 5,708,157 [IMAGE AVAILABLE] L3: 4 of 18

ABSTRACT:
Novel polynucleotides and the proteins encoded thereby are disclosed.

US PAT NO: 5,707,829 [IMAGE AVAILABLE] L3: 5 of 18
DATE ISSUED: Jan. 13, 1998
TITLE: DNA sequences and secreted proteins encoded thereby
INVENTOR: Kenneth Jacobs, Newton, MA
Kerry Kelleher, Marlborough, MA
McKeough Carlin, Cambridge, MA
John M. McCoy, Reading, MA
ASSIGNEE: Genetics Institute, Inc., Cambridge, MA (U.S. corp.)
APPL-NO: 08/514,014
DATE FILED: Aug. 11, 1995
ART-UNIT: 184
PRIM-EXMR: Robert A. Wax
ASST-EXMR: Nashaat T. Nashed
LEGAL-REP: Scott A. Brown, Thomas J. DesRosier

US PAT NO: 5,707,829 [IMAGE AVAILABLE] L3: 5 of 18

ABSTRACT:
Novel polynucleotides and the proteins encoded thereby are disclosed.

US PAT NO: 5,705,615 [IMAGE AVAILABLE] L3: 6 of 18
DATE ISSUED: Jan. 6, 1998
TITLE: **Antibodies** specific for HT.sub.m4
INVENTOR: Bing Lim, Dorchester, MA
Chaker N. Adra, Boston, MA
Jean-Michel Lelias, Columbus, OH
ASSIGNEE: Beth Israel Deaconess Medical Center, Boston, MA (U.S. corp.)
APPL-NO: 08/707,340
DATE FILED: Sep. 3, 1996
ART-UNIT: 186
PRIM-EXMR: Christina Y. Chan
ASST-EXMR: Emma Cech
LEGAL-REP: Hamilton, Brook, Smith & Reynolds, P.C.

US PAT NO: 5,705,615 [IMAGE AVAILABLE] L3: 6 of 18

ABSTRACT:
The invention relates to a recombinant DNA molecule which encodes a HT.sub.m4 protein, a transformed host cell which has been stably transfected with a DNA molecule which encodes a HT.sub.m4 protein and a recombinant HT.sub.m4 protein. The invention also relates to a method for detecting the presence of a hereditary atopy.

US PAT NO: 5,693,474 [IMAGE AVAILABLE] L3: 7 of 18
DATE ISSUED: Dec. 2, 1997
TITLE: Methods for cancer diagnosis and prognosis
INVENTOR: Jerry Shay, Dallas, TX
Michael David West, Belmont, CA
Woodring E. Wright, Arlington, TX
ASSIGNEE: Board of Regents, University of Texas System, Austin, TX

(U.S. corp.)
APPL-NO: 08/486,042
DATE FILED: Jun. 7, 1995
ART-UNIT: 187
PRIM-EXMR: Carla J. Myers
LEGAL-REP: Melya J. Hughes, Richard L. Neeley, Kevin R. Kaster

US PAT NO: 5,693,474 [IMAGE AVAILABLE] L3: 7 of 18

ABSTRACT:
A method for predicting tumor progression and prognosing cancer comprises: (a) collecting a sample suspected of containing cancer cells; (b) analyzing the sample for telomerase activity; (c) correlating the activity with a standard level of telomerase activity; and (d) correlating a high telomerase activity with an indication of unfavorable prognosis and a low telomerase activity with a favorable prognosis.

US PAT NO: 5,688,915 [IMAGE AVAILABLE] L3: 8 of 18
DATE ISSUED: Nov. 18, 1997
TITLE: Long term maintenance of lymphocytes in vitro
INVENTOR: Yakov Ron, East Brunswick, NJ
Joseph Dougherty, Hampton, NJ
ASSIGNEE: The University of Medicine and Dentistry of New Jersey, Newark, NJ (U.S. corp.)
APPL-NO: 08/457,482
DATE FILED: Jun. 1, 1995
ART-UNIT: 127
PRIM-EXMR: Nathan M. Nutter
LEGAL-REP: Klauber & Jackson

US PAT NO: 5,688,915 [IMAGE AVAILABLE] L3: 8 of 18

ABSTRACT:
Long term culture of resting T lymphocytes. The present invention provides methods and compositions for maintaining resting, mature T lymphocytes (cells) for long term in the absence of mitogens, antigens, or stimulatory cytokines, in which the T cells maintaining their ability to respond to nitrogens and allogenic cells. T cells cultured under such conditions can be used as target cells for retroviral vector-mediated gene transfer and implemented in certain gene therapy applications. The culturing conditions described herein allow for the continuous availability of T lymphocytes for various pharmacological, diagnostic, gene therapy, and experimental purposes, and can be utilized for any application requiring non-stimulated T lymphocytes. In specific examples, umbilical cord blood and peripheral blood T lymphocytes and endothelial-like adherent monocytes are cultured in DMEM, 2% 2-mercaptoethanol containing either 20% horse serum, or RPMI 2% 2-ME containing 10% fetal calf serum supplemented with basic fibroblast growth factor for up to three months.

US PAT NO: 5,658,761 [IMAGE AVAILABLE] L3: 9 of 18
DATE ISSUED: Aug. 19, 1997
TITLE: Stromal cell lines from human bone marrow and their use
INVENTOR: Karin Thalmeier, Munchen, Federal Republic of Germany
Peter Dormer, Gilching, Federal Republic of Germany

ASSIGNEE: Gsf-Forschungszentrum fur Umwelt und Gesundheit GmbH, OberscheleiBheim, Federal Republic of Germany (foreign corp.)
APPL-NO: 08/584,425
DATE FILED: Jan. 11, 1996
ART-UNIT: 185
PRIM-EXMR: James Ketter
LEGAL-REP: Nikaido, Marmelstein, Murray & Oram

US PAT NO: 5,658,761 [IMAGE AVAILABLE] L3: 9 of 18

ABSTRACT:
A human bone marrow stromal cell line, which is characterized in that the cells of the cell line, after irradiation which results in the growth being arrested, remain adherent, is suitable for use as a feeder layer for supporting the proliferation of blood cells.

US PAT NO: 5,654,173 [IMAGE AVAILABLE] L3: 10 of 18
DATE ISSUED: Aug. 5, 1997
TITLE: Secreted proteins and polynucleotides encoding them
INVENTOR: Kenneth Jacobs, Newton, MA
John M. McCoy, Reading, MA
Edward R. LaVallie, Tewksbury, MA
Lisa A. Racie, Acton, MA
David Merberg, Acton, MA
Maurice Treacy, Chestnut Hill, MA
Vikki Spaulding, Billerica, MA
ASSIGNEE: Genetics Institute, Inc., Cambridge, MA (U.S. corp.)
APPL-NO: 08/702,080
DATE FILED: Aug. 23, 1996
ART-UNIT: 181
PRIM-EXMR: Vasu S. Jagannathan
ASST-EXMR: Brian Lathrop
LEGAL-REP: Scott A. Brown, Thomas J. DesRosier

US PAT NO: 5,654,173 [IMAGE AVAILABLE] L3: 10 of 18

ABSTRACT:
Novel polynucleotides and the proteins encoded thereby are disclosed.

US PAT NO: 5,648,478 [IMAGE AVAILABLE] L3: 11 of 18
DATE ISSUED: Jul. 15, 1997
TITLE: Tissue-specific enhancer active in prostate
INVENTOR: Daniel R. Henderson, 769 Garland Dr., Palo Alto, CA 94303
APPL-NO: 08/380,916
DATE FILED: Jan. 30, 1995
ART-UNIT: 184
PRIM-EXMR: Jacqueline M. Stone
ASST-EXMR: D. Curtis Hogue, Jr.

US PAT NO: 5,648,478 [IMAGE AVAILABLE] L3: 11 of 18

ABSTRACT:
The invention provides a human prostate-specific transcriptional regulatory sequence, polynucleotide comprising such regulatory regions,

toxin gene constructs wherein a toxin gene is expressed under the transcriptional control of a human prostate-specific transcriptional regulatory sequence, and methods for treating prostate disease using such toxin gene constructs.

US PAT NO: 5,646,008 [IMAGE AVAILABLE] L3: 12 of 18
DATE ISSUED: Jul. 8, 1997
TITLE: Vertebrate **apoptosis** gene: compositions and methods
INVENTOR: Craig B. Thompson, Chicago, IL
Lawrence H. Boise, Chicago, IL
Gabriel Nunez, Ann Arbor, MI
ASSIGNEE: The Regent of the University of Michigan, Ann Arbor, MI (U.S. corp.)
APPL-NO: 08/081,448
DATE FILED: Jun. 22, 1993
ART-UNIT: 184
PRIM-EXMR: Keith C. Furman
LEGAL-REP: Arnold, White & Durkee

US PAT NO: 5,646,008 [IMAGE AVAILABLE] L3: 12 of 18

ABSTRACT:
The invention relates generally to compositions of and methods for obtaining and using a polypeptide other than BCL-2 that affects programmed vertebrate cell death. The invention relates as well to polynucleotides encoding those polypeptides, recombinant vectors carrying those sequences, the recombinant host cells including either the sequences or vectors, and recombinant polypeptides. The invention further provides methods for using the isolated, recombinant polypeptides in assays designed to select and improve substances capable of altering programmed cell death for use in diagnostic, drug design and therapeutic applications.

US PAT NO: 5,639,613 [IMAGE AVAILABLE] L3: 13 of 18
DATE ISSUED: Jun. 17, 1997
TITLE: Methods for cancer diagnosis and prognosis
INVENTOR: Jerry Shay, Dallas, TX
Michael David West, Belmont, CA
Woodring E. Wright, Arlington, TX
ASSIGNEE: Board of Regents, University of Texas System, Austin, TX (U.S. corp.)
APPL-NO: 08/423,403
DATE FILED: Apr. 18, 1995
ART-UNIT: 187
PRIM-EXMR: W. Gary Jones
ASST-EXMR: Carla Myers
LEGAL-REP: Melya J. Hughes, Richard L. Neeley, Kevin R. Kaster

US PAT NO: 5,639,613 [IMAGE AVAILABLE] L3: 13 of 18

ABSTRACT:
A method for predicting tumor progression and prognosing cancer comprises: (a) collecting a sample suspected of containing cancer cells; (b) analyzing the sample for telomerase activity; (c) correlating the activity with a standard level of telomerase activity; and (d)

correlating a high telomerase activity with an indication of unfavorable prognosis and a low telomerase activity with a favorable prognosis.

US PAT NO: 5,625,122 [IMAGE AVAILABLE] L3: 14 of 18
DATE ISSUED: Apr. 29, 1997
TITLE: Mouse having a disrupted lck gene
INVENTOR: Tak W. Mak, Toronto, Canada
ASSIGNEE: The Ontario Cancer Institute, Toronto, Canada (foreign corp.)
APPL-NO: 08/145,043
DATE FILED: Nov. 3, 1993
ART-UNIT: 184
PRIM-EXMR: Jacqueline M. Stone
ASST-EXMR: Deborah Crouch
LEGAL-REP: Marshall, O'Toole, Gerstein, Murray & Borun

US PAT NO: 5,625,122 [IMAGE AVAILABLE] L3: 14 of 18

ABSTRACT:
A mutant non-human mammal lacking expression of the lymphocyte-specific tyrosine kinase p56.sup.lck. Lck deficient mice possess few peripheral T lymphocytes and a pronounced thymic atrophy. The remaining thymus contains immature thymocytes surrounded by a perturbed thymic microenvironment. p56.sup.lck appears to play a crucial role in early thymocyte differentiation.

US PAT NO: 5,612,211 [IMAGE AVAILABLE] L3: 15 of 18
DATE ISSUED: Mar. 18, 1997
TITLE: Stimulation, production and culturing of hematopoietic progenitor cells by fibroblast growth factors
INVENTOR: Elaine L. Wilson, New York, NY
Janice Gabrilove, New York, NY
ASSIGNEE: New York University, New York, NY (U.S. corp.)
Sloan-Kettering Institute For Cancer Research, New York, NY (U.S. corp.)
APPL-NO: 08/076,875
DATE FILED: Jun. 15, 1993
ART-UNIT: 181
PRIM-EXMR: Christina Y. Chan
ASST-EXMR: David Lukton
LEGAL-REP: Browdy and Neimark

US PAT NO: 5,612,211 [IMAGE AVAILABLE] L3: 15 of 18

ABSTRACT:
Fibroblast growth factors are used in vivo, in situ and in vitro to stimulate stem cells, hemopoiesis, the immune system, transplant donor cells, culture and/or engraftment, wherein the use of fibroblast growth factors is disclosed for the stimulation of stem cells or hemopoietic cells, supporting cells and their progeny, in vitro, in situ and in vivo, as well as corresponding engrafting sites in vivo.

US PAT NO: 5,612,018 [IMAGE AVAILABLE] L3: 16 of 18
DATE ISSUED: Mar. 18, 1997

TITLE: Drug screening and treatment for HIV thymocyte depletion
INVENTOR: Mark L. Bonyhadi, Belmont, CA
Hideto Kaneshima, Palo Alto, CA
Joseph M. McCune, San Francisco, CA
Reiko Namikawa, Palo Alto, CA
Lishan Su, Palo Alto, CA
ASSIGNEE: Systemix, Inc., Palo Alto, CA (U.S. corp.)
APPL-NO: 08/183,178
DATE FILED: Jan. 18, 1994
ART-UNIT: 184
PRIM-EXMR: Jasemine C. Chambers
LEGAL-REP: Pamela J.Fish & Richardson P.C. Sherwood

US PAT NO: 5,612,018 [IMAGE AVAILABLE] L3: 16 of 18

ABSTRACT:
A method is provided for screening compounds for the ability to supress thymocyte depletion in thymuses of HIV-infected individuals, particularly enhancing the CD4.sup.+ -expressing population as compared to an untreated individual. Particularly, drugs are provided which allow for this result, cyclosporine A being exemplary.

US PAT NO: 5,521,067 [IMAGE AVAILABLE] L3: 17 of 18
DATE ISSUED: May 28, 1996
TITLE: Bone marrow cell adhesion molecules and process for detecting adherence between cell adhesion molecules and cells generally
INVENTOR: Beerelli Seshi, Fairport, NY
ASSIGNEE: University of Rochester, Rochester, NY (U.S. corp.)
APPL-NO: 08/158,936
DATE FILED: Nov. 24, 1993
ART-UNIT: 182
PRIM-EXMR: David Saunders
LEGAL-REP: Nixon, Hargrave, Devans & Doyle

US PAT NO: 5,521,067 [IMAGE AVAILABLE] L3: 17 of 18

ABSTRACT:
The present invention relates to proteins associated with human bone marrow cell membranes for adhering hematopoietic cells to human bone marrow cell membranes. These proteins are soluble in lithium dodecyl sulfate but insoluble in 2% nonaethylene glycol octylphenol ether (e.g., 2% Triton.RTM. X-100) solution. These proteins and **antibodies** raised against them are useful in the treatment and diagnosis of blood disorders. The DNA molecules encoding these proteins have use in gene therapy regimes. Also disclosed is a method for detecting binding between cell adhesion membrane proteins and cells having a potential to be bound to such proteins.

US PAT NO: 5,484,726 [IMAGE AVAILABLE] L3: 18 of 18
DATE ISSUED: Jan. 16, 1996
TITLE: **Antibodies** specific for human stromelysin-3 and a method for detection of stromelysin-3
INVENTOR: Paul Basset, Strasbourg, France
Jean-Pierre Bellocq, Strasbourg, France

Pierre Chambon, Bleasheim, France
ASSIGNEE: Bristol-Myers Squibb Company, Princeton, NJ (U.S. corp.)
Institute National de la Sante et de la Recherche Medicale , Paris Cedex, France (foreign govt.)
Centre National de la Recherche Scientifique, Paris Cedex, France (foreign govt.)
Universite Louis Pasteur, Strasbourg Cedex, France (foreign corp.)
APPL-NO: 08/001,711
DATE FILED: Jan. 7, 1993
ART-UNIT: 187
PRIM-EXMR: Margaret Parr
ASST-EXMR: Lisa Arthur
LEGAL-REP: Sterne, Kessler, Goldstein & Fox

US PAT NO: 5,484,726 [IMAGE AVAILABLE] L3: 18 of 18

ABSTRACT:
The present invention relates to a gene encoding stromylsin-3, which is a new member of the metalloproteinase family. Expression of the stromelysin-3 gene has been found to be specifically associated with invasive breast, head, neck and skin cancer. The invention also relates to **antibodies** which specifically bind to human stromylsin-3 and the use of these stromelysin-3 **antibodies** for detection of the stromylsin-3 protein in a sample.

=> s "Fas ligand" and bone marrow cells

```
          528 "FAS"
        18303 "LIGAND"
           11 "FAS LIGAND"
                ("FAS"(W)"LIGAND")
        30482 BONE
         5826 MARROW
       165497 CELLS
         1095 BONE MARROW CELLS
                (BONE(W)MARROW(W)CELLS)
L4          1 "FAS LIGAND" AND BONE MARROW CELLS
```

=> d l4 bib ab

US PAT NO: 5,756,086 [IMAGE AVAILABLE] L4: 1 of 1
DATE ISSUED: May 26, 1998
TITLE: Adenoviruses having modified fiber proteins
INVENTOR: Alan McClelland, Gaithersburg, MD
Susan C. Stevenson, Frederick, MD
ASSIGNEE: Genetic Therapy, Inc., Gaithersburg, MD (U.S. corp.)
APPL-NO: 08/591,492
DATE FILED: Feb. 6, 1996
ART-UNIT: 185
PRIM-EXMR: Johnny F. Railey, II
LEGAL-REP: Elliot M. Olstein, Raymond J. Lillie

US PAT NO: 5,756,086 [IMAGE AVAILABLE] L4: 1 of 1

ABSTRACT:

An adenovirus wherein the adenovirus fiber protein includes a ligand which is specific for a receptor located on a desired cell type. The adenovirus may have at least a portion of the adenovirus fiber protein removed and replaced with a ligand which is specific for a receptor located on a desired cell type, or the adenovirus may include a fusion protein of the adenovirus fiber protein and the ligand. Such an adenovirus may also include a gene(s) encoding a therapeutic agent(s) and may be "targeted" in order to deliver such gene(s) to a desired cell type.